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Remarks

Status of Claims

Applicants respectfully point out to the Examiner that the listing of claims as stated in Paper No. 13, on page 1, item 4a is incorrect. In response to the Restriction Requirement mailed on November 18, 2002, Applicants elected the claims of Group I (*i.e.*, claims 1-20, 29-52, 61-83, 92-115, and 124-127). Applicants also requested the rejoinder of Group II (*i.e.*, claims 21-28, 53-60, 84-91, and 116-123) and Group III (*i.e.*, claims 128-132), should the claims of Group I be found allowable. Subsequently, Applicants canceled claims 128-132 (Group III) in a Supplemental Response to Office Action mailed June 13, 2003.

Therefore, claims 1-20, 29-52, 61-83, 92-115, and 124-127 are under consideration and claims 21-28, 53-60, 84-91, and 116-123 are withdrawn. A proper listing of claims is shown on page 2, item 1 of Paper No. 13.

Withdrawn claim 53 has been amended to mirror claim 116. Support for the amendment can be found in the specification at, for instance, page 19, paragraph [0108]. Hence, no new matter was added by way of this amendment.

Claim rejection under 35 U.S.C. § 112, 2nd paragraph

Claim 92 is rejected under 35 U.S.C. § 112, second paragraph as allegedly indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. *See*, Paper No. 13, page 2, item 2. More particularly, the Examiner observes that “claim 92 recites the ATCC deposit No. 97103 rather than the ATCC Deposit No. 97157.” *See*, Paper No. 13, page 2, item 2.

Applicants thank the Examiner for pointing out this typographical error. Claim 92 has been amended accordingly.

In view of the above, Applicants believe the Examiner’s concerns have been fully addressed. Accordingly, Applicants respectfully request that the rejection under 35 U.S.C. § 112, second paragraph of claim 92 be reconsidered and withdrawn.

Claim rejection under 35 U.S.C. § 102(b)

Claims 1-2, 4, 8-10, 12, 16-17, 29-31, 34, 36, 40-42, 44, 48-49, 61-62, 65-66, 68, 72-74, 79-80, 92-94, 97, 99, 103-105, 107, 111-112, and 124-125 are rejected under 35 U.S.C. § 102(b) for allegedly being anticipated by Shau et al. (US Patent No. 5,250,295, later referred to as the “‘295 Patent”), in light of Shau et al. (US Patent No. 5,610,286, later referred to as the

“286 Patent”). *See*, Paper No. 13, page 2, item 3. More specifically, the Examiner states that the “antibody disclosed against a specific NKEF protein in the Shau et al ‘295 reference, anticipates the instant claims, because the reference protein is 68.8% identical to the instant protein of SEQ ID NO: 2. Therefore the antibodies of the Shau et al reference meet the limitations of an antibody as recited in instant claims 8-9 (*sic*).” *See*, Paper No. 13, page 3, paragraph 3. The Examiner further states “Shau et al. (US Pat No. 5,610,286) reference is relied upon because it teaches the amino acid sequence of NKEF C protein (see SEQ ID NO:2, column 14, lines 22-30), the amino acid sequence being an inherent characteristic of the protein.” *See*, Paper No. 13, page 3, second paragraph (emphasis added). Applicants respectfully disagree and traverse.

Preliminarily, Applicants assume that the Examiner’s remarks were intended to apply to the entire set of rejected claims and not only to claims 8-9. Therefore, Applicants address the present rejection as it applies to claims 1-2, 4, 8-10, 12, 16-17, 29-31, 34, 36, 40-42, 44, 48-49, 61-62, 65-66, 68, 72-74, 79-80, 92-94, 97, 99, 103-105, 107, 111-112, and 124-125.

Regarding the rejection of claims under 35 U.S.C. § 102(b), the M.P.E.P states that:

“[A] claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference.” *Verdegaal Bos. V. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQD2d 1051, 1053 (Fed. Cir. 1987). “The identical invention must be shown in as complete detail as is contained in the ... claim.” *Richardson v. Suzuki Motor Co.*, 868 F.2d 1226, 1236, 9 USPQD2d 1913, 1920 (Fed. Cir. 1989).

M.P.E.P., 8th Edition, § 2131 (August 2001). Anticipation can only be established by a single prior art reference that discloses each and every element of the claimed invention. *See Scripps Clinic & Research Found. v. Genentech, Inc.*, 927 F.2d 1565, 1576, 18 U.S.P.Q.2d 1001, 1010 (Fed. Cir. 1991), *clarified on recons.*, 18 U.S.P.Q.2d 1896 (Fed. Cir. 1991). In the absence of an express description of each and every element of the invention in a reference, *i.e.*, where a reference is silent about an asserted inherent characteristic of the claimed invention, inherent anticipation can only be established by showing: (1) that the inherent characteristic must necessarily be present in the prior art reference, and (2) that such characteristic would have to have been recognized by a person of ordinary skill in the art at the time. *See Glaxo Inc. v. Novopharm Ltd.*, 52 F.3d 1043, 1047 34 U.S.P.Q.2d 1565 (Fed. Cir. 1995); *Continental Can Co. USA Inc. v. Monsanto Co.*, 948 F.2d 1264, 20 U.S.P.Q.2d 1746, 1749 (Fed. Cir. 1991); *Mickowski v. Visi-Trak Corp.*, 36 F.Supp.2d 171 (S.D. N.Y. 1999).

Applicants respectfully assert that the polypeptides disclosed in the '286 Patent and the NKEF C polypeptide of the invention are distinct proteins. In support of this assertion, Applicants point to the specification where the two polypeptides NKEF A and B disclosed in the '286 Patent are compared to the polypeptide of the invention. *See*, specification at page 2, paragraph [0006]. Applicants further direct the Examiner's attention to Figures 2A to 2D of the application which represent an amino acid sequence alignment between the NKEF A, B, and C polypeptides as well as several other members of the family of antioxidant proteins to which NKEF A, B, and C belong. Furthermore, Applicants respectfully point to the amino acid sequence alignment between NKEF A (SEQ ID NO: 2 of the '286 Patent) and the NKEF C polypeptide of the invention provided by the Examiner in the pending Office Action. All this evidence demonstrates that NKEF A and C exhibit two distinct amino acid sequences.

Additionally, Applicants respectfully submit that the pending claims are directed at antibodies, or portion thereof, that specifically bind the polypeptide of the invention, or portion thereof. Applicants have distinguished above the NKEF C polypeptide of the invention from the NKEF A and B proteins disclosed in the '286 Patent. Consequently, Applicants assert that antibodies which specifically bind the NKEF C polypeptide of the invention would not recognize the NKEF A or B polypeptides.

The amino acid sequence alignment provided by the Examiner further shows that NKEF A and NKEF C share certain small portions of identical sequence. However, Applicants assert that the antibodies directed against those portions of amino acid sequence which are identical between NKEF A and NKEF C would not be specific to NKEF C as they would recognize other NKEF factors.

If an antibody binds essentially equally well both to the NKEF C protein of the invention and, for example, to another member of the NKEF family such as those shown in Figures 2A-2D of the application, then it no longer would be considered by one of skill in the art as an antibody that specifically binds to the NKEF C polypeptide, but rather would be considered as a cross reactive antibody. (*See*, Golub, E.S. and Green, D.R. *Immunology: a Synthesis*, 2nd edition, Sunderland, MA: Sinauer Associates, Inc. 1991: p. 27, attached herewith as Exhibit A: "Antibody molecules can exhibit great specificity, but there are cross-reactions – cases in which antibody to antigen A also reacts with antigen B. This can be due to the presence of the same molecular configuration, or antigenic determinant [epitope], on the two antigens." Cross-reacting antibodies are not the ones encompassed by the current claims, as the claims are directed to an antibody that "specifically binds" to a protein whose sequence is

SEQ ID NO:2 (or variants thereof) or to a protein encoded by the human cDNA contained in ATCC Deposit No. 97157 (or variants thereof) of the present invention.

Applicants submit that the phrase "specifically binds" is a term of art which is routinely used, recognized, and understood by those of ordinary skill in the antibody arts. The specificity of antibodies is defined in *Immunology: a Synthesis* by Edward Golub and Douglas Green (2nd edition, Sunderland, MA: Sinauer Associates, Inc. 1991: p. 23, attached herewith as Exhibit A) as "the ability of antibodies produced in response to an antigen to react with that antigen and not with others." Those of skill in the art commonly refer to antibodies as being specific, or as specifically binding particular antigens, without further explanation. For example, routine assays for identifying a "specific" antibody were available as of the priority date of the present application. *Current Protocols in Immunology*, a common laboratory handbook, provides three such assays: (1) Indirect ELISA to Detect *Specific* Antibodies; (2) Double Antibody-Sandwich ELISA to Detect *Specific* Antibodies; and (3) Double-Immunodiffusion Assay for Detecting *Specific* Antibodies (see, e.g., *Current Protocols in Immunology*-ed. Coligan *et al.* Vol. 2, Sections 2.1.1-2.1.20 and 2.3.1-2.3.3 (1991), attached hereto as Exhibit B (emphasis added). These or similar assays could readily be used by one of ordinary skill in the art to determine, without undue experimentation, if antibodies specifically bind to a polypeptide.

Those of ordinary skill in the art know that an antibody that "specifically binds" a polypeptide of the invention is an antibody that 1) will bind the polypeptide of SEQ ID NO:2 of the invention and the polypeptide encoded by the cDNA clone contained in ATCC Deposit Number 97157, as these polypeptides exist in either denatured or native forms; 2) may also bind fragments or variants of the polypeptides of the invention, such as splice variants, allelic variants, species orthologues¹ or muteins, depending on the presence or absence of the specific antibody's epitope in the fragment or variant; and 3) would not significantly bind paralogs¹ of the polypeptides of the invention or other non-related polypeptides.

Applicants assert that one of skill in the art would find adequate support in the specification for the preparation, isolation, and characterization of antibodies specific to NKEF C. For instance, on page 21, paragraphs [0117]-[0120] of the specification, several types of antibodies and methods to obtain them are described. Furthermore, on page 19,

¹ Orthologues are defined by the National Center for Biotechnology (NCBI) as: genes derived from a common ancestor through vertical descent. This is often stated as the same gene in different species. In contrast, paralogs are genes within the same genome that have evolved by duplication. (See the NCBI Field Glossary available online at <http://www.ncbi.nlm.nih.gov/Class/FieldGuide/glossary.html#A>, a printout of which is attached here with as Exhibit C.

paragraph [0108], an ELISA assay is described, which uses “an antibody specific to the NKEF C antigen, preferably a monoclonal antibody.” Using such assays as ELISA, Western Blot, radioimmunoassay, or competitive binding assay, one of ordinary skill in the art would be able to select the antibodies which recognize only NKEF C to the exception of any other members of the family of antioxidant proteins.

Thus, Applicants have provided ample support and description for the preparation and identification of antibodies directed solely to the NKEF C polypeptide, which would not recognize, for instance, the NKEF A and NKEF B polypeptides described in the ‘286 Patent. Therefore, the polyclonal antibodies described in the ‘286 Patent cannot anticipate the claimed antibodies of the invention because they are not antibodies which recognize specifically the NKEF C polypeptide of the invention.

As for the Examiner’s assertion that “Shau et al. (US Pat No. 5,610,286) reference is relied upon because it teaches the amino acid sequence of NKEF C protein (see SEQ ID NO:2, column 14, lines 22-30), the amino acid sequence being an inherent characteristic of the protein”, Applicants submit that the Shau *et al.* references, even when considered together, do not teach the nucleotide and corresponding amino acid sequences of the NKEF C polypeptide of the invention, either explicitly or inherently. First, the ‘295 Patent teaches the purification of one NKEF protein, whose identity does not correspond to NKEF C, based on comparison of the peptide sequences shown in column 7 of the ‘295 Patent (SEQ ID NOS: 1-3) to the amino acid sequence of NKEF C. The ‘295 Patent also teaches the production of an immune serum against this protein (*see*, the ‘295 Patent, column 6, lines 10-22 and 59-68, respectively). Second, the ‘286 Patent teaches the use of this immune serum in the isolation and characterization of two NKEF genes which are also distinct from NKEF C, and whose “cDNAs fell into one of two [categories] of closely related but non-identical genes, referred to as NKEF A and B.” *See*, the ‘286 Patent, column 14, lines 31-34. Additionally, according to the Examiner’s own alignment, the NKEF A disclosed in the ‘286 Patent is not identical to the NKEF C of the invention. Consequently, Applicants assert that the NKEF polypeptides isolated in the ‘295 and ‘286 Patents are not the NKEF C polypeptide of the invention. Therefore, the references cited by the Examiner do not anticipate the current claims because they do not teach each and every element of the claims, more particularly, the sequence of NKEF C polynucleotide and corresponding polypeptide.

Therefore, Applicants submit that one of skill in the art would recognize that the claimed antibodies are specific to the NKEF C protein of the invention to the exception of

non-specific antibodies. Thus, the claimed antibodies cannot be anticipated by the polyclonal antibodies directed against NKEF A and B described in the '286 Patent because only antibodies specifically recognizing NKEF C are claimed.

In view of the remarks submitted herewith, Applicants believe that the rejections of the Examiner have been overcome. Applicants respectfully request reconsideration and withdrawal of the rejection of claims 1-2, 4, 8-10, 12, 16-17, 29-31, 34, 36, 40-42, 44, 48-49, 61-62, 65-66, 68, 72-74, 79-80, 92-94, 97, 99, 103-105, 107, 111-112, and 124-125 under 35 U.S.C. § 102(b).

Claim rejection under 35 U.S.C. § 103(a)

A. Claims 3, 11, 35, 43, 67, 75, 85, 98, 106, and 117 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Shau *et al.* (See, above, later referred to as “Shau”), in view of Lerner (1982, later referred to as “Lerner”), and Harlow *et al.* (1988, later referred to as “Harlow”). See, Paper No. 13, page 4, item 4a. Applicants respectfully disagree and traverse.

In order for a rejection under 35 U.S.C. § 103(a) to be valid, three criteria must be met:

- (a) there must be some suggestions or motivation to modify or to combine reference teachings;
- (b) there must be a reasonable expectation of success;
and
- (c) the prior art reference (or references when combined) must teach or suggest all the claim limitations

(See, M.P.E.P. 706.02(j)) (emphasis added).

Applicants assert that neither the combination of Shau, Lerner, and Harlow nor the references individually disclose all the limitations of the present invention. Specifically, as shown above in response to the rejection of claims under 35 U.S.C. § 102(b), the Shau reference does not disclose antibodies that specifically bind to the NKEF C polypeptide of the invention. Accordingly, Applicants respectfully request that the rejection of claims 3, 11, 35, 43, 67, 75, 85, 98, 106, and 117 under 35 U.S.C. § 103(a) be withdrawn.

B. Claims 5-7, 13-15, 37-39, 45-47, 69-71, 76-78, 87-89, 100-102, 108-110, and 119-125 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Shau *et al.* (See,

above), in view of Queen (US Patent No. 5,530,101, later referred to as “Queen”). *See*, Paper No. 13, page 5, item 4b. Applicants respectfully disagree and traverse.

Applicants assert that neither the combination of Shau and Queen nor the references individually disclose all the limitations of the present invention. Specifically, as shown above in response to the rejection of claims under 35 U.S.C. § 102(b), the Shau reference does not disclose antibodies that specifically bind the NKEF C polypeptide of the invention. Accordingly, Applicants respectfully request that the rejection of claims 5-7, 13-15, 37-39, 45-47, 69-71, 76-78, 87-89, 100-102, 108-110, and 119-125 under 35 U.S.C. § 103(a) be withdrawn.

C. Claims 18-20, 50-52, 81-83, 100-102, and 113-115 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Shau *et al.* (*see*, above), in view of Lerner (1982, later referred to as “Lerner”), and Harlow *et al.* (1982, later referred to as “Harlow”) as applied to claims 3, 11, 35, 43, 67, 75, 85, 98, 106, and 117 above, further in view of Servier *et al.* (1981) (later referred to as “Servier”). *See*, Paper No. 13, page 6, item 4c. Applicants respectfully disagree and traverse.

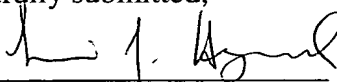
Applicants assert that neither the combination of Shau, Lerner, Harlow, and Servier, nor the references individually disclose all the limitations of the present invention. Specifically, as shown above in response to the rejection of claims under 35 U.S.C. § 102(b), the Shau reference does not disclose antibodies that specifically bind the NKEF C polypeptide of the invention. Accordingly, Applicants respectfully request that the rejection of claims 18-20, 50-52, 81-83, 100-102, and 113-115 under 35 U.S.C. § 103(a) be withdrawn.

Conclusion

If there are any fees due in connection with the filing of this paper, please charge the fees to our Deposit Account No. 08-3425. If a fee is required for an extension of time under 37 C.F.R. § 1.136 not accounted for above, such an extension is requested and the fee should also be charged to our Deposit Account.

Dated: November 24, 2003

Respectfully submitted,

By 

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Exhibit A

IMMUNOLOGY

A SYNTHESIS

Second Edition

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NOTE

PART-OPENING ELECTRON MICROGRAPHS

Part 1 (page 19): Antibody-hapten complex (purified rabbit anti-2,4-dinitro-phenyl antibody and a bivalent hapten). [From Valentine and Green, 1967. *J. Mol. Biol.* 27: 615]

Part 2 (page 191): A resting lymphocyte, probably a T cell ($\times 21,800$). [Courtesy of D. Zucker-Franklin, New York University Medical Center]

Part 3 (page 543): Immune complexes, seen as electron-dense, hump-shaped deposits in the upper third of the photo, along a capillary wall in a glomerulus following streptococcal glomerulonephritis ($\times 17,250$). [Courtesy of M. N. Yum, Indiana University Medical Center]

IMMUNOLOGY: A SYNTHESIS Second Edition

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free hapten. In general, carriers are molecules that are of themselves immunogenic. Hence we may think of the hapten as an added determinant on an already immunogenic molecule. The study of hapten-carrier systems has given us much information about the nature of antigens and the antigen-antibody interaction, but it has also been one of the keys to understanding the cellular events in the immune response (see Chapter 17).

The Specificity of Serological Reactions

Landsteiner studied haptens and carriers in an attempt to work out the rules that govern antigenicity. The compilation of these studies appeared in his classic treatise, *The Specificity of Serological Reactions*. As we will see, no universal rules governing antigenicity came out of this work, but what did emerge was the realization that the chemical properties of the antigen molecule determine the specificity of the immune system. SPECIFICITY is defined as the ability of antibodies produced in response to an antigen to react with that antigen and not with others. The thoroughness of Landsteiner's approach and the elegance of his thought make browsing in this volume, which is available in paperback, a worthwhile experience for any scientist.

Landsteiner immunized a rabbit with a hapten-carrier conjugate. This injection resulted in antiserum with both anti-hapten and anti-carrier activity. He then conjugated the hapten to a different carrier and reacted the conjugate with the same antiserum to test for the presence of anti-hapten antibodies. Because he had changed carrier molecules for the test, there was no anti-carrier reaction; the reaction observed was between the anti-hapten antibodies and the hapten. He then varied the properties of the hapten in order to study, for example, the effect of acidic or ionic groups on the ability of the antibody raised against the original hapten to react with the modified hapten. Although no general rules emerged, it is instructive to look at some of Landsteiner's conclusions (Landsteiner, 1962):

The principal results of numerous precipitin tests with azoproteins were the following ...

1. First of all, the nature of the acidic groups was of decisive influence. [p. 163]

Data from Landsteiner's experiments are shown in Tables 1-4. Antibody is raised against aminobenzene or aminobenzene with

Cross Reactivity

Antibody molecules can exhibit great specificity, but there are CROSS REACTIONS—cases in which antibody to antigen A also reacts with antigen B. This can be due to the presence of the same molecular configuration, or ANTIGENIC DETERMINANT, on the two antigens, or to properties of a determinant that allow it to be recognized as though it were another group. Antigenic determinants are also called *epitopes*. As we move through the book we will use these terms almost interchangeably. We can conceive of molecules that have similar but not identical structures and appear in closely related species. These molecules may have enough similarity to allow antibodies against one to react with the other.¹

Table 5 shows the percentage of cross reactivity between albumins of different species. Antibody was made against bovine serum albumin (BSA), and the extent of the ability of albumins from other species to react with the anti-BSA was then determined. This cross reactivity is probably due to the presence of common determinants on the different albumins. To determine this, however, each of the determinants must be isolated and studied chemically. Even then, as we will see later in this chapter, we cannot be quite certain of

¹ The neurobiologist A. K. Hall has suggested the term IMMUNOFREQUENT for such determinants.

Table 5 Cross reaction between BSA and other albumins.*

Albumin source	Percentage of cross reactivity with BSA	Albumin source	Percentage of cross reactivity with BSA
Human	15	Mouse	10
Pig	32	Rat	13
Sheep	25	Hamster	13
Horse	13	Cat	25
Guinea pig	13	Vallaroo	6
Dog	13		

Source: Data from Weigle, 1961. *J. Immunol.* 37: 599.

*Rabbit anti-BSA was absorbed with each of the albumins listed and then tested for its ability to react with BSA. This ability is expressed as a percentage cross reactivity. The data show that sheep BSA has the highest amount of cross reactivity and guinea pig and vallaroo the least.

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All assays for antibody production depend upon the measurement of the interaction of elicited antibody with antigen. This binding of antigen by antibody can be measured in solution by methods such as equilibrium dialysis or fluorescence quenching, by measuring the production of insoluble antigen-antibody complexes in aqueous solutions or gels, or by measuring the adsorption of soluble antigen to solid-phase antibody (or conversely, the binding of soluble antibody to solid-phase antigen). It is far beyond the scope of this manual, and unnecessary in any but a historical context, to consider even a large sampling of the available methods for measuring antibodies or antigens. For modern practical purposes, most investigators require methods that are rapid, sensitive, specific, quantitative (at least in terms of comparison to some known standard), and reproducible. Ideally, the methods should not require particularly expensive reagents or equipment, and they should be effective for measuring both polyclonal antibodies as well as monoclonal antibodies. These methods should also be useful in evaluating antigens when known amounts of specific antibodies are available.

This section describes two general methods for assaying antibodies and antigens, representative of both extremes of chronological development—the enzyme-linked immunosorbent assay (ELISA; *UNITS 2.1 & 2.2*), a highly versatile, sensitive, and quantitative technique that requires little equipment and for which critical reagents are readily available, and the double-immunodiffusion assay (*UNIT 2.3*), which requires nothing more than a glass slide, Pasteur pipet, agar, and phosphate-buffered saline. Both assays are useful for evaluating antibody activity of polyclonal antisera, but by and large, the double-immunodiffusion assay is not useful for evaluating binding of monoclonal antibodies to monovalent antigens. The versatility of ELISAs is emphasized by the six distinct ELISA protocols presented in *UNIT 2.1*: an indirect ELISA to detect specific antibodies, a direct competitive ELISA to detect soluble antigens, an antibody-sandwich ELISA to detect soluble antigens, a double antibody-sandwich ELISA to detect specific antibodies, and two cellular ELISAs, one to detect cell-surface antigens, and one to detect antibodies specific for surface antigens. The advantages of each of these assays for particular applications are summarized in Table 2.1.1. Many additional variations exist, and the specific needs of a particular problem may be better served by minor changes and adaptations of these protocols; a particular adaptation of the antibody-sandwich ELISA for isotyping antibodies is presented in *UNIT 2.2*.

Other methods described elsewhere in this manual may also be effectively employed as assays for antibodies or antigens. Direct or indirect immunofluorescence (using cytofluorimetry) of appropriately selected cell lines or tissues can be used to obtain both qualitative and quantitative information about the binding of an antibody or antiserum (*UNITS 5.1-5.4*). In addition, antibodies specifically required for either immunoprecipitation (*UNIT 8.3*) or immunoblotting (*UNIT 8.10*), including antipeptide antibodies, may be best assayed by these same methods. However, as long as a carefully designed assay is chosen for a particular antibody or antiserum, the ultimate result will be not only a high-quality reagent, but a quantitative and sensitive means of identifying the antigen in question. The ability to identify new antigens with carefully raised antibodies is limited only by the investigator's imagination and diligence.

Enzyme-Linked Immunosorbent Assays

This unit describes six different ELISA systems for the detection of specific antibodies, soluble antigens, or cell-surface antigens. In all six systems, soluble reactants are removed from solution after specifically binding to solid-phase reactants. Table 2.1.1 summarizes the different ELISA protocols, which are illustrated in Figures 2.1.1-2.1.6.

In the first four protocols, solid-phase reactants are prepared by adsorbing an antigen or antibody onto plastic microtiter plates; in the next two protocols, the solid-phase reactants are cell-associated molecules. In all protocols, the solid-phase reagents are incubated with secondary or tertiary reactants covalently coupled to an enzyme. Unbound conjugates are washed out and a chromogenic or fluorogenic substrate is added.

Table 2.1.1 Summary of ELISA Protocols

ELISA protocol	Uses	Required reagents	Comments
Indirect	Antibody screening; epitope mapping	Antigen, pure or semipure; test solution containing antibody; enzyme conjugate that binds Ig of immunized species	Does not require the use of preexisting specific antibodies; requires relatively large amounts of antigen
Direct competitive	Antigen screening; detect soluble antigen	Antigen, pure or semipure; test solution containing antigen; enzyme-antibody conjugate specific for antigen	Rapid assay with only two steps; excellent for measuring antigenic cross-reactivity
Antibody-sandwich	Antigen screening; detect soluble antigen	Capture antibody (purified or semi-purified specific antibody); test solution containing antigen; enzyme-antibody conjugate specific for antigen	Most sensitive antigen assay; requires relatively large amounts of pure or semi-pure specific antibody (capture antibody)
Double antibody-sandwich	Antibody-screening; epitope mapping	Capture antibody: (specific for Ig of immunized species); test solution containing antigen; enzyme-antibody conjugate specific for antigen	Does not require purified antigen; relatively long assay with five steps
Direct cellular	Screen cells for expression of antigen; measure cellular antigen expression	Cells that express antigen of interest; enzyme-antibody conjugate specific for cellular antigen	Sensitive assay for bulk screening; insensitive to heterogeneity of expression in mixed population of cells
Indirect cellular	Screen for antibodies against cellular antigens	Cells used for immunizing; test solution containing antibodies; enzyme conjugate that binds Ig of immunized species	May not detect antibodies specific for cellular antigens expressed at a low density

As the substrate is hydrolyzed by the bound enzyme conjugate, a colored or fluorescent product is generated. Finally, the product is detected visually or with a microtiter plate reader. The amount of product generated is proportional to the amount of analyte in the test mixture. The first support protocol can be used to optimize the different ELISAs. The second support protocol presents a method for preparing alkaline phosphatase conjugates.

INDIRECT ELISA TO DETECT SPECIFIC ANTIBODIES

BASIC PROTOCOL

This assay is useful for screening antisera or hybridoma supernatants for specific antibodies when milligram quantities of purified or semi-purified antigen are available (1 mg of purified antigen will permit screening of 80 to 800 microtiter plates; Fig. 2.1.1). Antibodies are detected by coating the wells of microtiter plates with antigen, incubating the coated plates with test solutions containing specific antibodies, and washing away unbound antibodies. A solution containing a developing reagent, (e.g., alkaline phosphatase conjugated to protein A, protein G, or antibodies against the test solution antibodies) is then added to the plate. After incubation, unbound conjugate is washed away and substrate solution is added. After a second incubation, the amount of substrate hydrolyzed is assessed with a spectrophotometer or spectrofluorometer. The measured amount is proportional to the amount of specific antibody in the test solution. Visual inspection can also be used to detect hydrolysis.

Materials

Developing reagent: protein A-alkaline phosphatase conjugate (Sigma #P9650), protein G-alkaline phosphatase conjugate (Calbiochem #539304), or anti-Ig-alkaline phosphatase conjugate (second support protocol)

Antigen solution

PBS (APPENDIX 2) containing 0.05% NaN_3 (PBSN)

Water, deionized or distilled

Blocking buffer

Test antibody samples

4-methylumbelliferyl phosphate (MUP) or *p*-nitrophenyl phosphate (NPP)
substrate solution

0.5 M NaOH (optional)

Multichannel pipet and disposable pipet tips

Immulon 2 (Dynatech #011-010-3450), Immulon 4 (Dynatech #011-010-3850), or equivalent microtiter plates

Plastic squirt bottles

Microtiter plate reader (optional)—spectrophotometer with 405-nm filter or spectrofluorometer (Dynatech #011-970-1900) with 365-nm excitation filter and 450-nm emission filter

Determine developing reagent and antigen concentrations

1. Determine the optimal concentration of the developing reagent (conjugate) by criss-cross serial-dilution analysis (see first support protocol).

Good conjugates of many specificities are available commercially. Choice of developing reagent (i.e., conjugate) is determined by the goals of the assay. If it is necessary to detect all antibodies that bind to antigen, conjugates prepared with antibodies specific for Ig κ and λ light chains should be used. Alternatively, protein A- or protein G-enzyme conjugates may be preferable when screening monoclonal antibodies. Specific monoclonal antibodies that bind protein A or protein G are easy to purify and characterize.

Antibody Detection
and Preparation

2.1.3

2. Determine the final concentration of antigen coating reagent by criss-cross serial-dilution analysis (see first support protocol). Prepare an antigen solution in PBSN at this final concentration. The final concentration of antigen is usually 0.2 to 10.0 $\mu\text{g/ml}$. Prepare ~6 ml antigen solution for each plate.

Pure antigen solution concentrations are usually $\leq 2 \mu\text{g/ml}$. Although pure antigen preparations are not essential, >3% of the protein in the antigen solution should be the antigen. The total concentration of protein in the antigen solution should be increased for semipurified antigen preparations. Do not raise the total protein concentration in the antigen solution to $>10 \mu\text{g/ml}$, since this concentration usually saturates >85% of the available sites on Immulon microtiter plates. For some antigens, coating may occur more efficiently at different pHs.

Coat plate with antigen

3. Using a multichannel pipet and tips, dispense 50 μl antigen solution into each well of an Immulon microtiter plate. Tap or shake the plate to ensure that the antigen solution is evenly distributed over the bottom of each well.

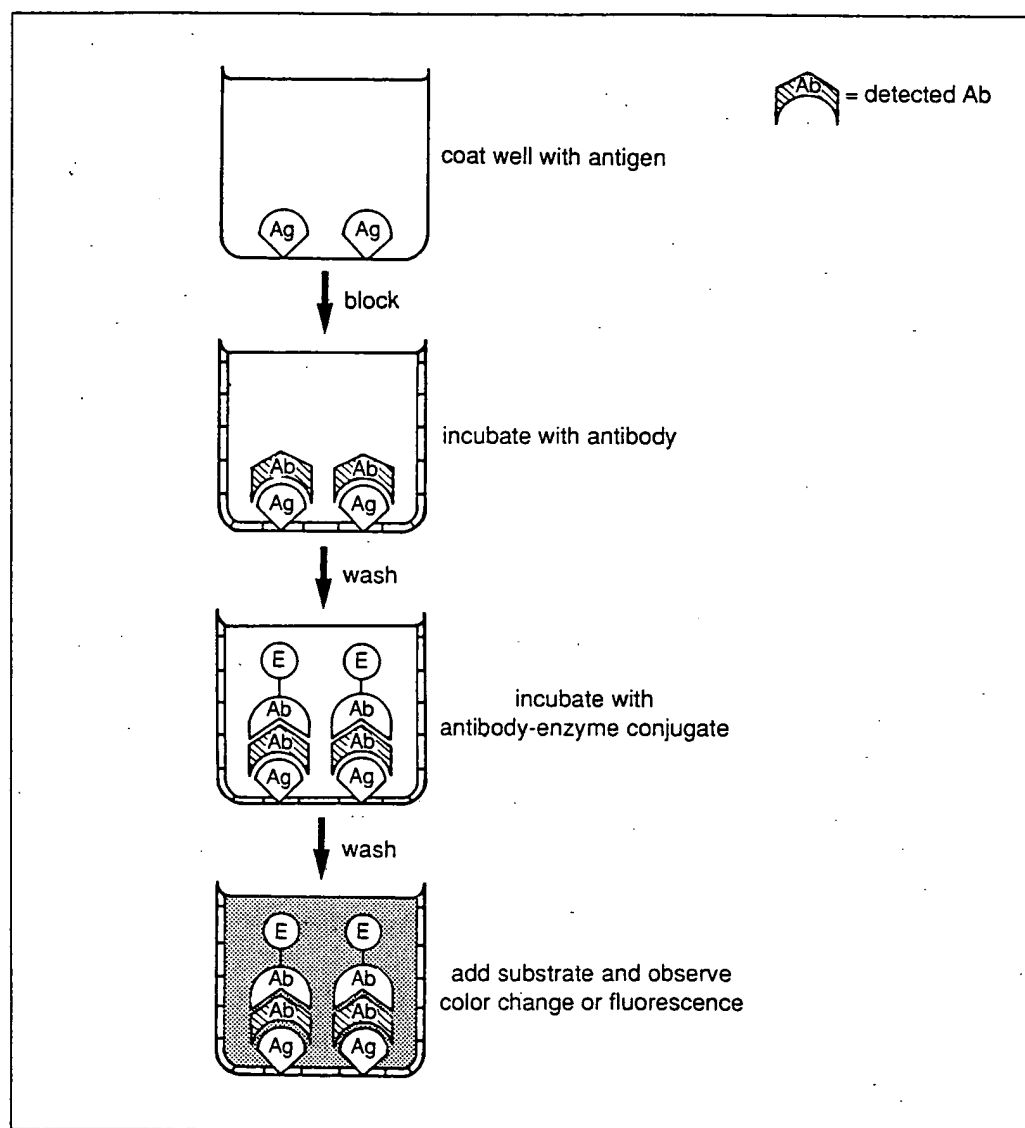


Figure 2.1.1 Indirect ELISA to detect specific antibodies. Ag = antigen; Ab = antibody; E = enzyme.

4. Wrap coated plates in plastic wrap to seal and incubate overnight at room temperature or 2 hr at 37°C.

Individual adhesive plate sealers are sold commercially but plastic wrap is easier to use and works as well. Sealed plates can be stored at 4°C with antigen solution for months.

5. Rinse coated plate over a sink by filling wells with deionized or distilled water dispensed either from a plastic squirt bottle or from the tap. Flick the water into the sink and rinse with water two more times, flicking the water into the sink after each rinse.

Block residual binding capacity of plate

6. Fill each well with blocking buffer dispensed as a stream from a squirt bottle and incubate 30 min at room temperature.

Residual binding capacity of the plate is blocked in this step. Tween 20 (0.05%) by itself is more effective at blocking than any protein tested, but because the combination of protein and Tween 20 may be more effective than Tween 20 alone in some cases, bovine serum albumin (BSA; 0.25%) is included in the blocking buffer.

7. Rinse plate three times in water as in step 5. After the last rinse, remove residual liquid by wrapping each plate in a large paper tissue and gently flicking it face down onto several paper towels laying on the benchtop.

Rinsing with water is cheaper and easier than rinsing with buffered solutions and is as effective.

Add antibody to plate

8. Add 50 µl antibody samples diluted in blocking buffer to each of the coated wells, wrap plate in plastic wrap, and incubate ≥2 hr at room temperature.

While enough antibody may be bound after 1 to 2 hr to generate a strong signal, equilibrium binding is generally achieved after 5 to 10 hr. Thus, the specific signal may be significantly increased by longer incubations.

For this and all steps involving the delivery of aliquots of many different solutions to microtiter plates with multichannel pipets, such as the primary screening of hybridoma supernatants, the same pipet tips can be reused for hundreds of separate transfers. Wash tips between transfers by expelling any liquid remaining in the tips onto an absorbent surface of paper tissues, rinsing tips five times in blocking buffer, and carefully expelling any residual liquid from tips onto the tissues. Avoid bubbles in the tips; any tip with intractable bubbles should be replaced.

Wash the plate

9. Rinse plate three times in water as in step 5.
10. Fill each well with blocking buffer, vortex, and incubate 10 min at room temperature.
Plates are vortexed to remove any reagent remaining in the corners of the wells.
11. Rinse three times in water as in step 5. After the final rinse, remove residual liquid as in step 7.

Add developing reagent to plate

12. Add 50 µl developing reagent in blocking buffer (at optimal concentration determined in step 1) to each well, wrap in plastic wrap, and incubate ≥2 hr at room temperature.

The strength of the signal may be increased by longer incubations (see annotation to step 8).

13. Wash plates as in steps 9 to 11.

After final rinsing, plates may be wrapped in plastic wrap and stored for months at 4°C prior to adding substrate.

Add substrate and measure hydrolysis

14. Add 75 μ l MUP or NPP substrate solution to each well and incubate 1 hr at room temperature.
15. Monitor hydrolysis qualitatively by visual inspection or quantitatively with a microtiter plate reader (see below). Hydrolysis can be stopped by adding 25 μ l of 0.5 M NaOH.
 - a. Visually, hydrolysis of NPP can be detected by the appearance of a yellow color. If using a microtiter plate reader to measure NPP hydrolysis, use a 405-nm filter.
 - b. Visually, hydrolysis of MUP can be monitored in a darkened room by illumination with a long-wavelength UV lamp. If using a microtiter plate spectrofluorometer to measure MUP hydrolysis, use a 365-nm excitation filter and a 450-nm emission filter.

The fluorogenic system using the MUP substrate is 10 to 100 times faster than the chromogenic system using NPP. Furthermore, the rate of spontaneous hydrolysis of MUP is much lower than that of NPP.

To detect bound antibodies that are present at low concentration, measure hydrolysis at a later time. To calculate when to measure hydrolysis the second time, remember that the amount of hydrolysis is almost directly proportional to the time of hydrolysis. For example, if the hydrolysis in the wells of interest reads 200 at 1 hr and a reading of 2000 is desired, incubate the plate ~10 hr before taking the second reading.

ALTERNATE PROTOCOL

DIRECT COMPETITIVE ELISA TO DETECT SOLUBLE ANTIGENS

This assay is used to detect or quantitate soluble antigens and is most useful when both a specific antibody and milligram quantities of purified or semi-purified antigen are available (Fig. 2.1.2). To detect soluble antigens, plates are coated with antigen and the binding of specific antibody-enzyme conjugates to antigen-coated plates is inhibited by test solutions containing soluble antigen. After incubation with mixtures of the conjugate and inhibitor in antigen-coated wells, unbound conjugate is washed away and substrate is added. The amount of antigen in the test solutions is proportional to the inhibition of substrate hydrolysis and can be quantitated by interpolation onto an inhibition curve generated with serial dilutions of a standard antigen solution.

The direct assay may also be adapted as an indirect assay by substituting specific antibody for specific antibody-enzyme conjugate. The amount of specific antibody bound is then detected using a species-specific or isotype-specific conjugate as a tertiary reactant.

Additional Materials

- Specific antibody-alkaline phosphatase conjugate (second support protocol)
- Standard antigen solution
- Test antigen solutions
- Round- or cone-bottom microtiter plates

1. Determine the optimal concentration of coating reagent and antibody-alkaline phosphatase conjugate by criss-cross serial-dilution analysis in which the concentrations of both the antigen (coating reagent) and the conjugate (developing reagent) are

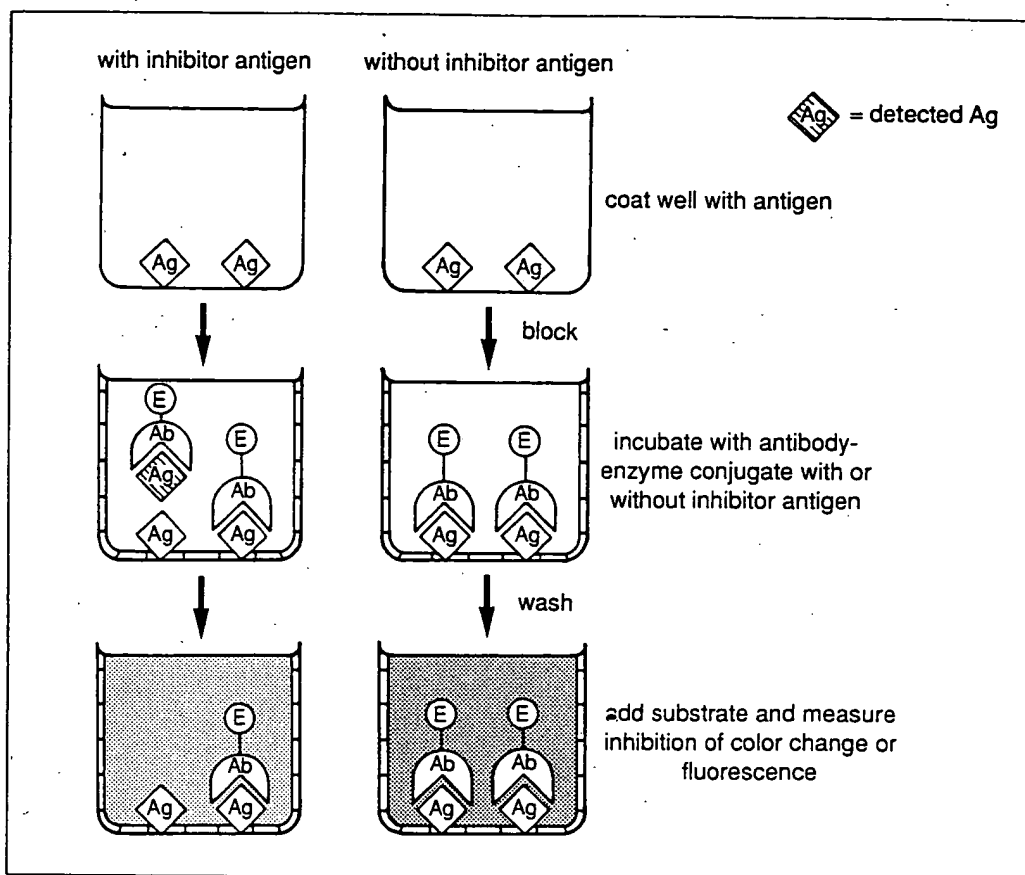


Figure 2.1.2 Direct competitive ELISA to detect soluble antigens. Ag = antigen; Ab = antibody; E = enzyme.

varied (see first support protocol). Prepare a 2× conjugate solution by diluting the specific antibody–alkaline phosphatase conjugate in blocking buffer to twice the optimal concentration.

The final concentration is usually 25 to 500 ng antibody/ml. Prepare 3 ml antibody–alkaline phosphatase conjugate for each plate.

2. Coat and block wells of an Immulon microtiter plate with 50 μ l antigen solution as in steps 2 to 7 of the basic protocol.
3. Prepare six 1:3 serial dilutions of standard antigen solution in blocking buffer (see first support protocol for preparation of serial dilutions)—these antigen concentrations will be used in preparing a standard inhibition curve (see step 10).

Antigen concentrations should span the dynamic range of inhibition. The dynamic range of inhibition is defined as that range of inhibitor concentrations wherein changes of inhibitor concentration produce detectable changes in the amount of inhibition. The dynamic range of inhibition is empirically determined in an initial assay in which antigen concentration is typically varied from the micromolar (10^{-6} M) to the picomolar (10^{-12} M) range. For most protein antigens, initial concentration should be ~ 10 μ g/ml, followed by nine 1:4 serial dilutions in blocking buffer. These antigen dilutions are assayed for their ability to inhibit the binding of conjugate to antigen-coated plates under standard assay conditions. From this initial assay, six 1:3 antigen dilutions spanning the dynamic range of inhibition are selected for further use as standard antigen-inhibitor dilutions. Prepare ≥ 75 μ l of each dilution for each plate to be assayed.

Inhibitor curves are most sensitive in the region of the curve where small changes in inhibitor concentrations produce maximal changes in the amount of inhibition. This

region of the curve normally spans 15% to 85% inhibition. In most systems, this range of inhibition is produced by concentrations of inhibitor between 1 and 250 ng/ml.

4. Mix and incubate conjugate and inhibitor by adding 75 μ l of 2 \times conjugate solution (from step 1) to each well of a round- or cone-bottom microtiter plate, followed by 75 μ l inhibitor—either test antigen solution or standard antigen solution (from step 3). Mix the conjugate and inhibitor solutions by pipetting up and down in the pipet tip three times (see annotation to step 8 in the basic protocol) and incubate \geq 30 min at room temperature.

For accurate quantitation of the amount of antigen in the test solutions, test antigen solutions should inhibit conjugate binding between 15% to 85%. It may be necessary to assay two or three different dilutions of the test solutions to produce inhibitions within this range.

5. Prepare uninhibited control samples by mixing equal volumes of 2 \times conjugate solution and blocking buffer.
6. Transfer 50 μ l of the mixture of conjugate plus inhibitor (from step 4) or conjugate plus blocking buffer (from step 5) to an antigen-coated plate (from step 2) and incubate 2 hr at room temperature.

If samples are to be assayed in duplicate, the duplicates should be in adjacent columns on the same plate. Reserve column 11 for uninhibited control samples (step 5) and column 12 for substrate alone without any conjugate. If the concentration of antigen in the test samples is to be accurately quantitated, dilutions of homologous antigen solutions (step 3) should be included on each plate.

7. Wash plate as in steps 9 to 11 of the basic protocol.
8. Add 75 μ l of MUP or NPP substrate solution to each well and incubate 1 hr at room temperature.
9. Read plates on the microtiter plate reader after \geq 1 hr, at which time enough substrate has been hydrolyzed in the uninhibited reactions to permit accurate measurement of the inhibition.
10. Prepare a standard antigen-inhibition curve constructed from the inhibitions produced by the dilutions of the standard antigen solutions from step 3. Plot antigen concentration on the x axis, which is a log scale, and fluorescence or absorbance on the y axis, which is a linear scale.
11. Interpolate the concentration of antigen in the test solutions from the standard antigen-inhibition curve.

The dynamic range of the inhibition curve may deviate from linearity if the specific antibodies are heterogeneous and possess significantly different affinities or if the standard antigen preparation contains heterogeneous forms of the antigen. Antigen concentration in test samples can be accurately interpolated from the inhibition curve as long as the test antigen is antigenically identical to the standard antigen and the concentration of test antigen falls within the dynamic range of inhibition.

ANTIBODY-SANDWICH ELISA TO DETECT SOLUBLE ANTIGENS

ALTERNATE PROTOCOL

Antibody-sandwich ELISAs may be the most useful of the immunosorbent assays for detecting antigen because they are frequently between 2 and 5 times more sensitive than those in which antigen is directly bound to the solid phase (Fig. 2.1.3). To detect antigen, the wells of microtiter plates are coated with specific (capture) antibody followed by incubation with test solutions containing antigen. Unbound antigen is washed out and a different antigen-specific antibody conjugated to enzyme (i.e., developing reagent) is added, followed by another incubation. Unbound conjugate is washed out and substrate is added. After another incubation, the degree of substrate hydrolysis is measured. The amount of substrate hydrolyzed is proportional to the amount of antigen in the test solution.

Additional Materials

Specific antibody or immunoglobulin fraction from antiserum or ascites fluid, or hybridoma supernatant (UNIT 2.6)

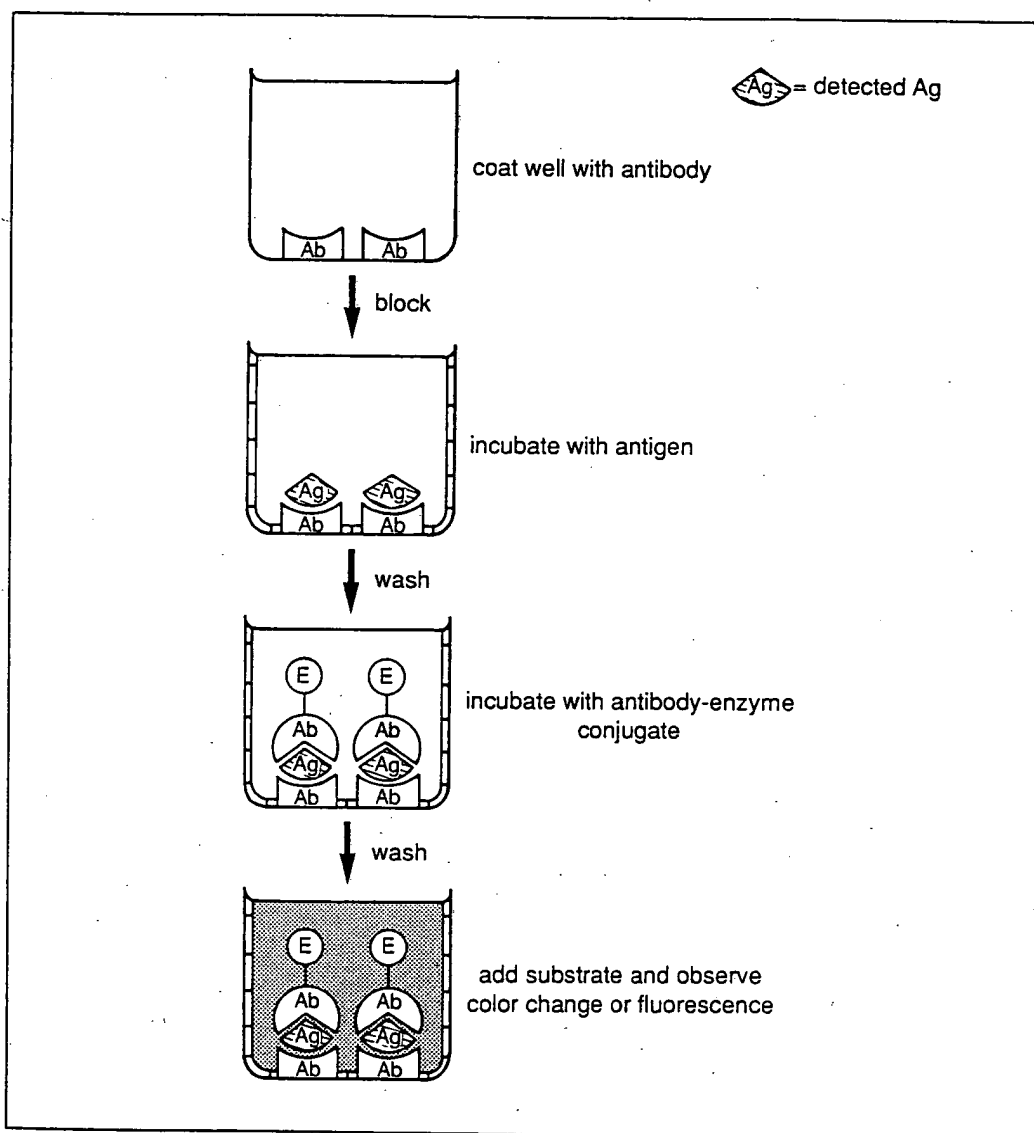


Figure 2.1.3 Antibody-sandwich ELISA to detect antigen. Ag = antigen; Ab = antibody; E = enzyme.

1. Prepare the capture antibody by diluting specific antibody or immunoglobulin fraction in PBSN to a final concentration of 0.2 to 10 $\mu\text{g/ml}$.

The capture antibodies can be monoclonal or polyclonal.

If the immunoglobulin fraction from an antiserum or ascites fluid is used, the concentration of total protein may need to be increased to compensate for the lower content of specific antibody. Little advantage is gained by increasing the total protein concentration in the capture antibody solution beyond 10 $\mu\text{g/ml}$.

2. Determine the concentration of capture antibody and conjugate necessary to detect the desired concentration of antigen by criss-cross serial-dilution analysis (see first support protocol). Prepare a capture antibody solution in PBSN at this concentration.
3. Coat wells of an Immulon plate with capture-antibody solution as in steps 3 to 5 of the basic protocol.
4. Block wells as in steps 6 and 7 of the basic protocol.
5. Prepare a standard antigen-dilution series by successive 1:3 dilutions of the homologous antigen stock in blocking buffer (see first support protocol).

In order to measure the amount of antigen in a test sample, the standard antigen-dilution series needs to span most of the dynamic range of binding. This range typically spans from 0.1 to 1000 ng antigen/ml. The dynamic range of binding is defined as that range of antigen concentrations wherein small, incremental changes in antigen concentration produce detectable differences in the amount of antigen bound (see annotation to step 3, in the preceding alternate protocol). In most assay systems, the amount of antigen in a test solution is most accurately interpolated from the standard curve if it produces between 15% to 85% of maximal binding.

Note: While standard curves are necessary to accurately measure the amount of antigen in test samples, they are unnecessary for qualitative "yes/no" answers.

6. Prepare dilutions of test antigen solutions in blocking buffer.

It may be necessary to assay one or two serial dilutions of the initial antigen test solution to ensure that at least one of the dilutions can be accurately measured. For most assay systems, test solutions containing 1 to 100 ng/ml of antigen can be accurately measured.

7. Add 50- μl aliquots of the antigen test solutions and the standard antigen dilutions (from step 5) to the antibody-coated wells and incubate ≥ 2 hr at room temperature.

For accurate quantitation, samples should be run in duplicate or triplicate, and the standard antigen-dilution series should be included on each plate (see step 5). Pipetting should be performed rapidly to minimize differences in time of incubation between samples.

8. Wash plate as in steps 9 to 11 of the basic protocol.
9. Add 50 μl specific antibody-alkaline phosphatase conjugate and incubate 2 hr at room temperature.

The conjugate concentration is typically 25 to 400 ng specific antibody/ml.

When the capture antibody is specific for a single determinant, the conjugate must be prepared from antibodies which recognize different determinants that remain available after the antigen is bound to the plate by the capture antibody.

10. Wash plate as in steps 9 to 11 of the basic protocol.
11. Add 75 μl of MUP or NPP substrate solution to each well and incubate 1 hr at room temperature.

12. Read the plate on a microtiter plate reader.

To quantitate low-level reactions, the plate can be read again after several hours of hydrolysis.

13. Prepare a standard curve constructed from the data produced by serial dilutions of the standard antigen (step 5). Plot antigen concentration on the x axis which is a log scale, and fluorescence or absorbance on the y axis which is a linear scale.
14. Interpolate the concentration of antigen in the test solutions from the standard curve.

DOUBLE ANTIBODY-SANDWICH ELISA TO DETECT SPECIFIC ANTIBODIES

ALTERNATE PROTOCOL

This assay is especially useful when screening for specific antibodies in cases when a small amount of specific antibody is available and purified antigen is unavailable (Fig. 2.1.4). Additionally, this method can be used for epitope mapping of different monoclonal antibodies that are directed against the same antigen. Plates are coated with capture antibodies specific for immunoglobulin from the immunized species. The test antibody solution is incubated on the plates coated with the capture antibodies. Plates are then washed, incubated with antigen, washed again, and incubated with specific antibody conjugated to an enzyme. After incubation, unbound conjugate is washed out and substrate is added. Wells that are positive for hydrolysis may contain antibodies specific for the antigen.

Additional Materials

Capture antibodies specific for immunoglobulin from the immunized species
Specific antibody-alkaline phosphatase conjugate

1. Coat wells of an Immulon microtiter plate with 50 μ l of 2 to 10 μ g/ml capture antibodies as in steps 2 to 5 of the basic protocol.

NOTE: Capture antibodies must not bind the antigen or conjugate antibodies. When analyzing hybridoma supernatants or ascites fluid, coat plates with 2 μ g/ml capture antibody. When analyzing antisera, coat plates with 10 μ g/ml capture antibody.

2. Block wells as in steps 6 and 7 of the basic protocol.
3. Prepare dilutions of test antibody solutions in blocking buffer. Add 50 μ l to coated wells and incubate ≥ 2 hr at room temperature.

Hybridoma supernatants, antisera, or ascites fluid can be used as the test samples. Dilute hybridoma supernatants 1:5 and antisera or ascites fluid 1:200.

4. Wash plate as in steps 9 to 11 of the basic protocol.
5. Prepare an antigen solution in blocking buffer containing 20 to 200 ng/ml antigen.
Although purified antigen preparations are not essential, the limit of detectability for most protein antigens in this type of system is 2 to 20 ng/ml. A concentration of 20 to 200 ng antigen/ml is recommended.
6. Add 50- μ l aliquots of the antigen solution to antibody-coated wells and incubate ≥ 2 hr at room temperature.
7. Wash plate as in steps 9 to 11 of the basic protocol.

8. Add 50 μ l specific antibody-alkaline phosphatase conjugate to the wells and incubate 2 hr at room temperature.

The conjugate antibodies must not react with the capture antibody or the test antibody. The conjugate concentration is typically between 25 to 500 ng specific antibody/ml, and should be high enough to result in ~0.50 absorbance units/hr at 405 nm when using NPP as a substrate or a signal of 1000 to 1500 fluorescence units/hr when using MUP as a substrate. If no specific antibodies from the appropriate species

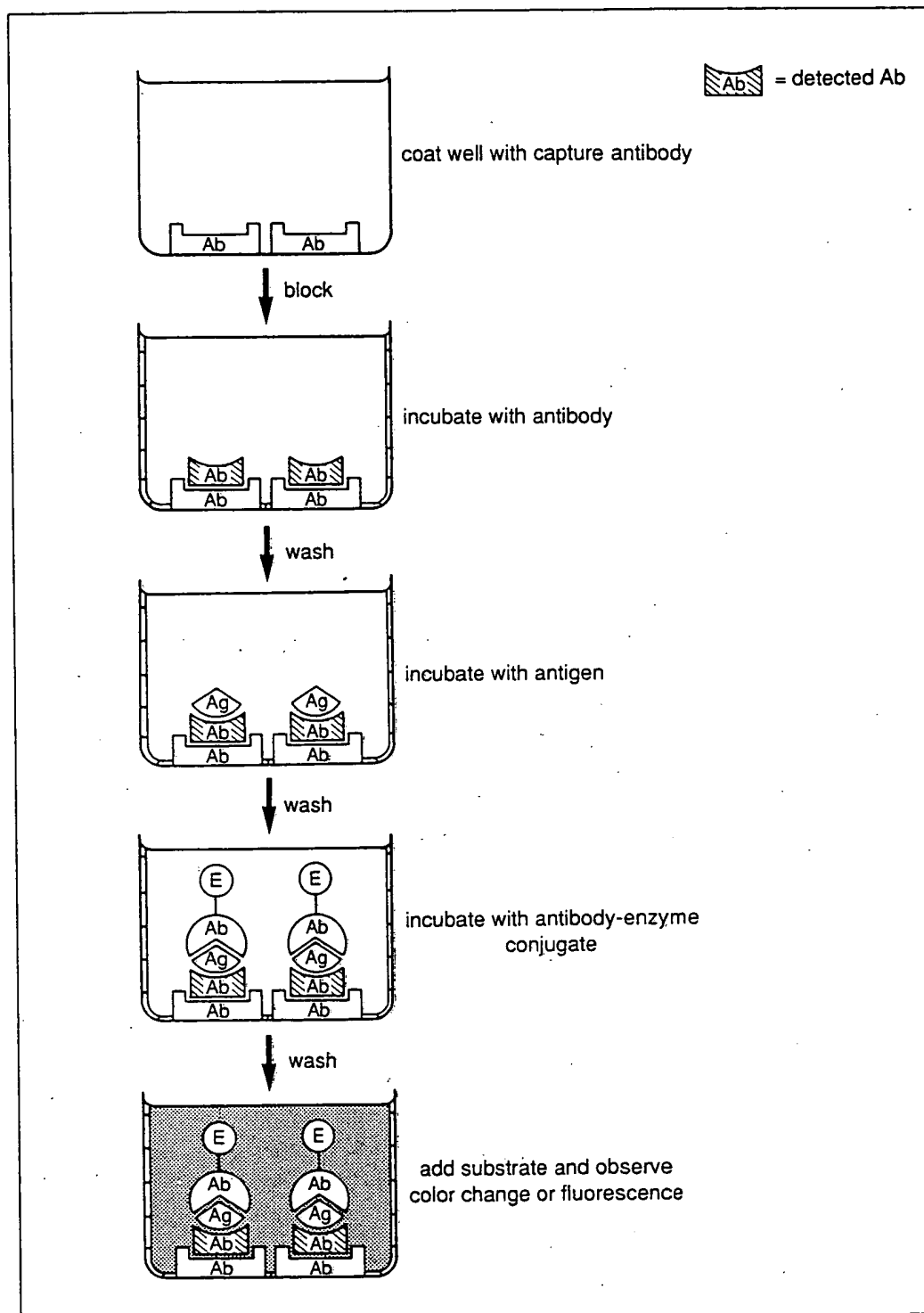


Figure 2.1.4 Double antibody-sandwich ELISA to detect specific antibodies. Ag = antigen; Ab = antibody; E = enzyme.

are available to serve as a positive control, then a positive control system should be constructed out of available reagents. Such reagents can be found in Linscott's *Directory of Immunological and Biological Reagents*.

9. Wash plate as in steps 9 to 11 of the basic protocol.
10. Add 75 μ l of MUP or NPP substrate solution to each well and incubate 1 hr at room temperature. After 1 hr, examine hydrolysis visually or spectrophotometrically (see step 15 of the basic protocol).

In order to detect low-level reactions, the plate can be read again after several hours or days of hydrolysis.

11. Check for false positives by rescreening samples that test positive for antigen-specific antibody. For each positive sample, coat four wells with capture antibody and arm the capture antibody with test antibody (steps 1 to 4). Incubate two of the wells with antigen (steps 5 to 7) and two of the wells with blocking buffer. Add conjugate and substrate to all four wells (steps 8 to 10) and measure hydrolysis after 1 hr.

This procedure will eliminate false positives resulting from test antibodies that react with the enzyme-antibody complex.

DIRECT CELLULAR ELISA TO DETECT CELL-SURFACE ANTIGENS

The expression of cell-surface antigens or receptors is measured using existing antibodies or other ligands specific for cell-surface molecules (Fig. 2.1.5). Cells are incubated with enzyme conjugated to antibodies that are specific for a cell-surface molecule. Unbound conjugate is washed away and substrate is added. The level of antigen expression is proportional to the amount of substrate hydrolysis. This procedure can be as sensitive as flow cytometry analysis in quantitating the level of antigen expression on a population of cells (UNITS 5.1 - 5.4). Unlike the flow cytometry analysis, however, this method is not sensitive for mixed populations. This assay can be converted to an indirect assay by substituting biotinylated antibody for the enzyme-antibody conjugate, followed by a second incubation with avidin-alkaline phosphatase.

ALTERNATE PROTOCOL

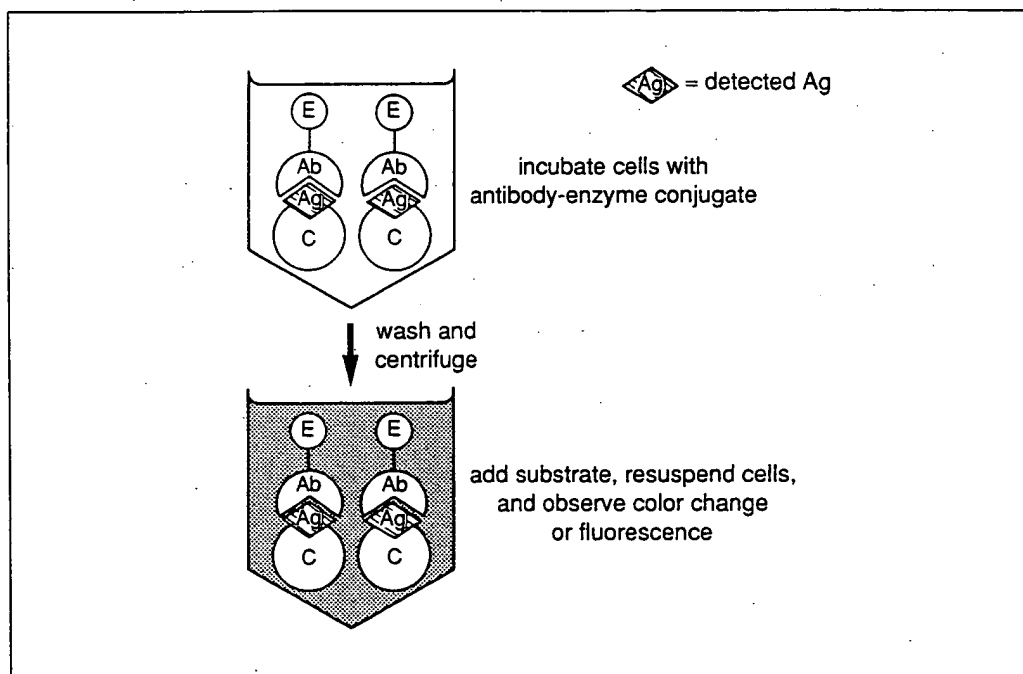


Figure 2.1.5 Direct cellular ELISA to detect cell-surface antigens. Ab = antibody; E = enzyme; C = cell.

Antibody Detection
and Preparation

2.1.13

Additional Materials

Cell samples

Specific antibody-alkaline phosphatase conjugate (see second support protocol)

Wash buffer, ice-cold

Cone- or round-bottom microtiter plates

Sorvall H-1000B rotor (or equivalent)

1. Determine the optimal number of cells per well and the antibody-conjugate concentration by criss-cross serial-dilution analysis (see first support protocol) using variable numbers of positive- and negative-control cell samples and varying concentrations of antibody-biotin conjugate.

Titrate cells initially at $1-5 \times 10^5$ /well and conjugate at 0.5 to 10 μ g/ml. For preparation and handling of cells, consult steps 2 to 5.

Because eukaryotic cells express variable amounts of alkaline phosphatase, test cells must be assayed in a preliminary experiment for alkaline phosphatase by incubation with substrate alone. If the test cells express unacceptable levels of alkaline phosphatase, another enzyme conjugate such as β -galactosidase should be used. Both chromogenic and fluorogenic substrates are available for β -galactosidase.

2. Centrifuge cell samples in a table-top centrifuge 5 min in Sorvall H-1000B rotor at 1500 rpm ($450 \times g$), 4°C , in a 15- to 50-ml centrifuge tube. Count cells (APPENDIX 3) and resuspend in ice-cold wash buffer at $1-5 \times 10^6$ cells/ml.

If the surface antigen retains its antigenicity after fixation, cells may be fixed at the beginning of the experiment—but do not fix cells unless it can be demonstrated that the antigenicity is retained after fixation. Fix cells by suspending in glutaraldehyde (0.5% final; from a 25% stock, EM grade Sigma #G5882), and incubating 30 min at room temperature. Pellet cells, resuspend in PBSLE (see second support protocol), and incubate for 30 min at 37°C . Wash twice in PBSLE and resuspend in wash buffer. Cells can be kept for months at 4°C after fixation.

3. Dispense 100 μ l of cell suspension ($1-5 \times 10^5$ cells) into wells of cone- or round-bottom microtiter plates, and centrifuge 1 min at $450 \times g$, 4°C . Remove supernatant by vacuum aspiration, and disrupt pellet by briefly shaking microtiter plate on a vortex mixer or microtiter plate shaker.
4. Resuspend pellet in 100 μ l of conjugate in ice-cold wash buffer at the optimal concentration (see step 1). Incubate 1.5 hr at 4°C , resuspending cells by gently shaking at 15-min intervals.

Be careful not to splash cell suspensions out of wells.

5. Centrifuge cells 1 min at $450 \times g$, 4°C , remove supernatant by vacuum aspiration, briefly vortex pellet, and resuspend in 200 μ l ice-cold wash buffer. Repeat three times.
6. Add 100 μ l MUP or NPP substrate solution. Incubate 1 hr at room temperature, resuspending cells by gently shaking at 15-min intervals during hydrolysis.
7. Determine extent of hydrolysis by visual inspection or using a microtiter plate reader.

INDIRECT CELLULAR ELISA TO DETECT ANTIBODIES SPECIFIC FOR SURFACE ANTIGENS

ALTERNATE PROTOCOL

This assay is designed to screen for antibodies specific for cell-surface antigens (Fig. 2.1.6). Antibodies against surface antigens are detected by incubating whole cells with a test solution containing the primary antibody. The unbound antibody is washed away, and the cells are then incubated with an enzyme conjugated to antibodies specific for the primary antibody. Unbound enzyme conjugate is washed away and substrate solution added. The level of bound primary antibody is proportional to the amount of substrate hydrolysis.

Additional Materials

Positive-control antibodies (i.e., those that react with the experimental cells and are from the immunized species)

Negative-control antibodies (i.e., those that do not react with the experimental cells)

Test antibody solution

Antibody- or $F(ab)_2$ (against immunoglobulin from the immunized species)-alkaline phosphatase conjugate

Cone- or round-bottom microtiter plates

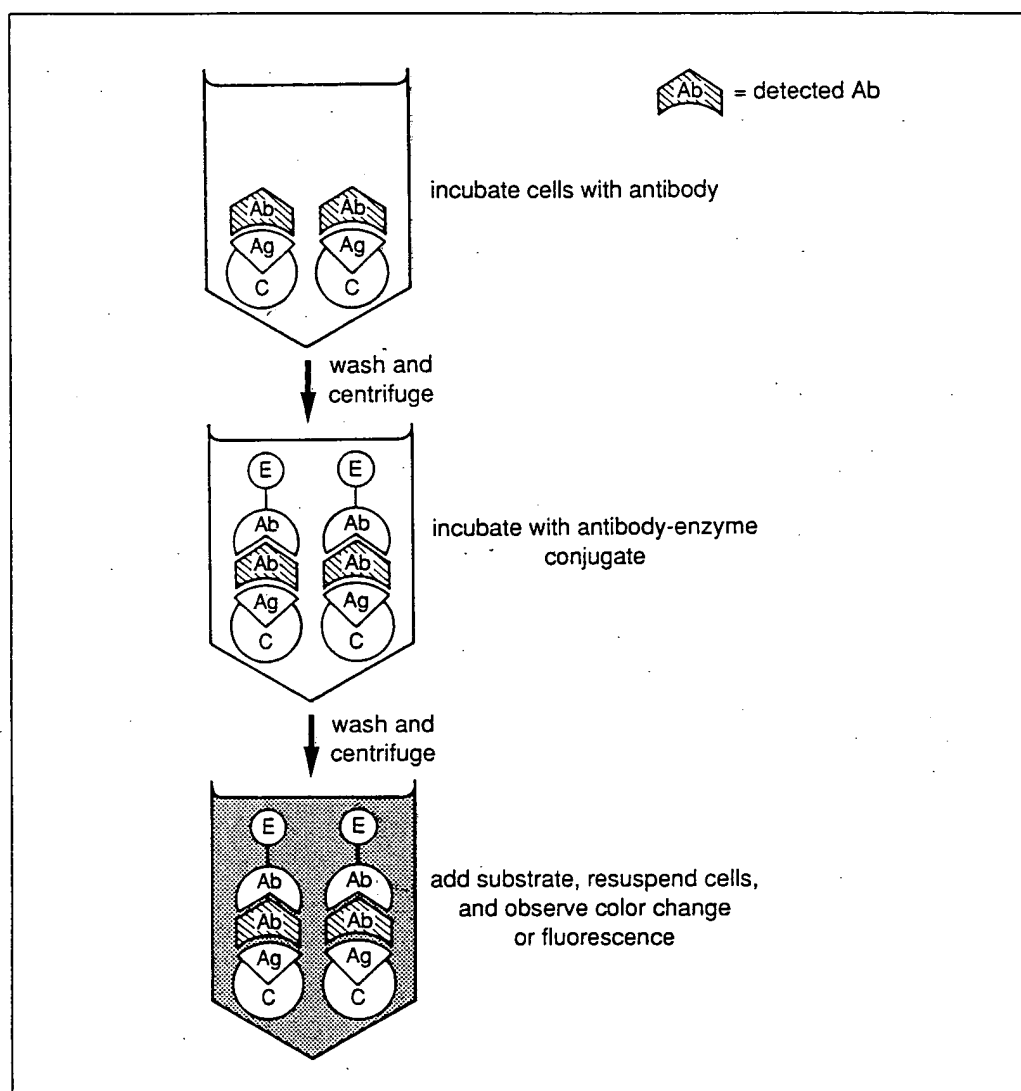


Figure 2.1.6 Indirect cellular ELISA to detect antibodies specific for surface antigens. Ab = antibody; E = enzyme; C = cell.

**Antibody Detection
and Preparation**

2.1.15

1. Centrifuge and resuspend cell samples as in step 2 of the previous alternate protocol at $1-5 \times 10^6$ cells/ml.

Because this technique detects antibodies against uncharacterized epitopes, fixation prior to analysis is not recommended. Fixation may destroy the antigenicity of the epitope. All steps must be performed at 4°C in physiological buffers containing NaN_3 . Because eukaryotic cells express variable amounts of alkaline phosphatase, test cells must be assayed for alkaline phosphatase activity. If the endogenous alkaline phosphatase level is too high, another enzyme should be substituted for alkaline phosphatase in the antibody-enzyme conjugate (see annotation to step 1 of the previous alternate protocol).

2. In preliminary assays, determine the optimal number of cells per well and conjugate concentration by criss-cross serial-dilution analysis using positive- and negative-control antibodies instead of test antibodies (see first support protocol). In adapting the criss-cross serial-dilution analysis, whole cells replace the solid-phase coating reagent; see techniques for handling cells are outlined in steps 3 to 8. Set up titrations by varying the number of cells between 1×10^5 and 5×10^5 /well, the concentration of positive- and negative-control antibodies between 0.1 and 10 $\mu\text{g/ml}$, and the concentration of antibody-enzyme conjugate between 0.1 and 10 $\mu\text{g/ml}$.
3. Dispense 100 μl of cell suspension ($1-5 \times 10^5$ cells) into wells of round- or cone-bottom microtiter plates. Centrifuge 1 min at 1500 rpm, 4°C, remove supernatant by vacuum aspiration, and disrupt pellet by briefly shaking microtiter plate on the vortex mixer.
4. Resuspend cells in 100 μl solutions containing 1 to 10 $\mu\text{g/ml}$ test antibody or control antibodies in ice-cold wash buffer. Incubate 1.5 hr at 4°C, resuspending cells by gently shaking at 15-min intervals.

Be careful not to splash cell suspensions out of wells.

5. Centrifuge cells 1 min at 1500 rpm, 4°C, remove supernatant by vacuum aspiration, briefly vortex pellet, and resuspend in 200 μl ice-cold wash buffer. Repeat twice.
6. Resuspend pellet in 100 μl enzyme-antibody conjugate or F(ab')_2 -enzyme conjugate diluted in ice-cold wash buffer. The optimal concentration of antibody, determined in step 2, is usually 100 to 500 ng/ml. Incubate 1.5 hr at 4°C, resuspending cells by gently shaking at 15-min intervals.

When working with cells that may express Fc receptors, it is best to use enzyme conjugated to F(ab')_2 fragments. F(ab')_2 fragments have had the Fc portion of the antibody enzymatically removed and no longer bind to Fc receptors.

7. Wash cells as in step 5. Repeat three times.
8. Add 100 μl MUP or NPP substrate solution. Allow hydrolysis to proceed until the signal has reached the desired levels; resuspend cells by gently shaking at 15 min intervals during hydrolysis. If desired, stop hydrolysis by adding 25 μl of 0.5 M NaOH.
9. Determine extent of hydrolysis by visual inspection or spectrophotometrically using a microtiter plate reader.

CRISS-CROSS SERIAL-DILUTION ANALYSIS TO DETERMINE OPTIMAL REAGENT CONCENTRATIONS

SUPPORT PROTOCOL

Serial dilution titration analyses are performed to determine optimal concentrations of reagents to be used in ELISAs. In this protocol, all three reactants in a three-step ELISA—a primary solid-phase coating reagent, a secondary reagent that binds the primary reagent, and an enzyme-conjugated tertiary developing reagent that binds to the secondary reagent—are serially diluted and analyzed by a criss-cross matrix analysis (Fig 2.1.7). Once the optimal concentrations of reagents to be used under particular assay conditions are determined, these variables are kept constant from experiment to experiment. The coating (primary), secondary, and developing (tertiary) reagents will vary depending upon which of the previous protocols needs to be optimized.

Additional Materials

Coating reagent
Secondary reagent
Developing reagent
17 × 100-mm and 12 × 74-mm test tubes

		Secondary reactant													
		homologous (antigen)						heterologous (antigen)							
(ng/ml)		200	50	12.5	3.12	0.78	0	200	50	12.5	3.12	0.78	0		
Tertiary reactant (antibody-alkaline phosphatase)	500	over	over	over	3200	1000	0	500	120	40	20	10		Rows	A
	250	over	over	over	2060	560	0	300	80	20	0	0			B
	125	over	over	3650	1370	360	0	195	40	10	10	0			C
	62.5	3600	4000	2270	790	240	0	120	30	10	10	10			D
	31.25	2700	2100	1200	410	120	0	60	10	10	10	0			E
	0	0	0	0	0	0	0	0	0	0	0	0			F
															G
															H
		1	2	3	4	5	6	7	8	9	10	11	12	Columns	

Figure 2.1.7 Results of a criss-cross serial-dilution analysis (for optimization of secondary and tertiary reactant concentrations) of an antibody-sandwich ELISA to detect antigen. The numbers in columns 1 to 11 and rows B to G represent relative fluorescence units observed for each well on a 96-well microtiter plate.

Plates were coated overnight with the capture antibody at 2 µg/ml. The secondary reactants, 4-fold serial dilutions of the homologous antigen and a non-cross-reactive heterologous antigen, were incubated on the plate 2 hr. The tertiary reactant, 2-fold serial dilutions of specific antibody-alkaline phosphatase conjugates, were incubated on the plate 2 hr. After 1 hr of incubation with the substrate MUP, the fluorescence was read in a microtiter plate spectrofluorometer.

Reagent concentrations depend upon individual assay variables that are set by the investigator. If the time of hydrolysis is set at 1 hr, the relative fluorescence at ~1000 relative fluorescence units, and the sensitivity at 780 pg/ml of homologous antigen, then 500 ng/ml of enzyme-antibody conjugate must be used in the ELISA. If, however, the assay has to detect only 3.12 ng/ml of homologous antigen, then the concentration of conjugate can be reduced to 125 ng/ml. It should be noted by comparing the homologous with the heterologous reactions (wells B5 versus B11 and D4 versus D10) that both the specificity and the signal-to-noise ratio for this assay are excellent.

Prepare coating-reagent dilutions

1. Place four 17 × 100-mm test tubes in a rack and add 6 ml PBSN to the last three tubes. In tube 1, prepare a 12-ml solution of coating reagent at 10 µg/ml in PBSN. Transfer 6 ml of tube 1 solution to tube 2. Mix by pipetting up and down five times. Repeat this transfer and mix for tubes 3 and 4; the tubes now contain the coating reagent at 10, 5, 2.5, and 1.25 µg/ml.
2. Using a multichannel pipet, dispense 50 µl of the coating reagent solutions into wells of four Immulon microtiter plates (i.e., each plate is filled with one of the four dilutions). Incubate overnight at room temperature or 2 hr at 37°C.
3. Rinse and block plates with blocking buffer as in steps 5 to 7 of the basic protocol.

Prepare secondary-reagent dilutions

4. Place five 12 × 75-mm test tubes in a rack and add 3 ml blocking buffer to the last four tubes. In tube 1, prepare a 4-ml solution of secondary reagent at 200 ng/ml in PBSN. Transfer 1 ml of tube 1 solution to tube 2. Pipet up and down five times. Repeat this transfer and mix for tubes 3 to 5; the tubes now contain the secondary reactant at 200, 50, 12.5, 3.125, and 0.78 ng/ml. If possible, prepare and test serial dilutions of a nonreactive heterologous form of the secondary reactant in parallel (Fig. 2.1.7).

If the assay is especially insensitive, it may be necessary to increase the secondary reactant concentrations so the tube-1 solution is 1000 ng/ml.

5. Dispense 50 µl of the secondary reagent solutions into the first five columns of all four coated plates. The most dilute solution is dispensed into column 5, while solutions of increasing concentration are added successively into columns 4, 3, 2, and 1. Thus, the fifth column contains 0.78 ng/ml and the first column 200 ng/ml. Incubate 2 hr at room temperature.
6. Wash plates as in steps 9 to 11 of the basic protocol.

Prepare developing-reagent dilutions

7. Place five 17 × 100-mm test tubes in a rack and add 3 ml blocking buffer to the last four tubes. In tube 1, prepare a 6-ml solution of developing reagent at 500 ng/ml in blocking buffer. Transfer 3 ml of tube 1 solution into tube 2 and mix. Repeat this transfer and mixing for tubes 3 and 4—the tubes now contain the developing reagent at 500, 250, 125, 62.5, and 31.25 ng/ml.
8. Dispense 50 µl of the developing reagent solutions into the wells of rows 2 to 6 of each plate, dispensing the most dilute solution into row 6 and solutions of increasing concentration successively into rows 5, 4, 3, and 2. Incubate 2 hr at room temperature.
9. Wash plates as in steps 9 to 11 of the basic protocol.

Measure hydrolysis

10. Add 75 µl MUP or NPP substrate solution to each well, incubate 1 hr at room temperature, and measure the degree of hydrolysis visually or with a microtiter plate reader. An appropriate assay configuration results in 0.50 absorbance units/hr at 405 nm when using NPP as a substrate or 1000 to 1500 fluorescence units/hr when using MUP as a substrate.

These results can be used to adjust optimal concentrations in the basic and alternate protocols.

PREPARATION OF ANTIBODY-ALKALINE PHOSPHATASE CONJUGATES

SUPPORT PROTOCOL

Antibodies are mixed with alkaline phosphatase and cross-linked by incubation with glutaraldehyde for 2 hr. The reaction is stopped by adding lysine and ethanolamine contained in PBSLE. The mixture is then desalted on a small Sephadex G-25 sizing column and the fractions are analyzed to detect those containing conjugate.

Additional Materials

- >0.2 mg/ml antibody in PBS
- Alkaline phosphatase in NaCl solution (Sigma #P0905)
- 25% glutaraldehyde, EM grade (Sigma #G5882)
- PBS containing 100 mM lysine and 100 mM ethanolamine (PBSLE)
- Blocking buffer containing 2.5 mM MgCl_2
- 10-ml Sephadex G-25 column (APPENDIX 3)
- 0.2- μm filter

1. Prepare a 1:3 mixture of antibody/alkaline phosphatase in PBS at >0.2 mg/ml total protein concentration.

Because of the high specific activity and long shelf-life of most antibody-alkaline phosphatase conjugates, an initial preparation of 0.5 mg antibody and 1.5 mg alkaline phosphatase will usually produce enough conjugate to analyze 200 to 800 microtiter plates.

2. Add 25% glutaraldehyde to 0.2% final while vortexing. Incubate 2 hr at room temperature. Stop reaction by adding an equal volume of PBSLE.
3. Desalt the sample by chromatography on a Sephadex G-25 column in PBSN; bed volume of the column should be 5 to 10 times larger than the reaction volume. Collect fractions that are one-half the volume of the reaction volume.
4. Assay fractions by transferring 2 μl into tubes containing 0.5 ml NPP substrate solution. Pool the first five fractions that strongly hydrolyze NPP.

While it is not essential to remove coupled from uncoupled reactants, this method will enrich for enzyme-antibody conjugates.

5. Mix the pool 1:2 in blocking buffer containing 2.5 mM MgCl_2 , filter through a 0.2- μm filter, and store at 4°C.

REAGENTS AND SOLUTIONS

Borate-buffered saline (BBS)

- 0.17 M H_3BO_4
- 0.12 M NaCl
- Adjust to pH 8.5 with NaOH

Blocking buffer

BBS (see above) containing:

- 0.05% Tween 20
- 1 mM EDTA
- 0.25% bovine serum albumin (BSA)
- 0.05% NaN_3
- Store at 4°C

Gelatin may be substituted for BSA; 5% instant milk has been successfully used but may interfere nonspecifically with antibody binding.

Antibody Detection
and Preparation

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MUP substrate solution

0.2 mM 4-methylumbelliferyl phosphate (MUP; Sigma #M8883)

0.05 M NaCO₃

0.05 mM MgCl₂

Store at room temperature

NPP substrate solution

3 mM *p*-nitrophenyl phosphate (NPP; Sigma #104-0)

0.05 M NaCO₃

0.05 mM MgCl₂

Store at 4°C

Test antibody solution

Hybridoma supernatants (UNIT 2.6) can usually be diluted 1:5 and ascites fluid and antisera (UNIT 2.4) diluted 1:500 in blocking buffer and still generate a strong positive signal. Dilutions of nonimmune ascites or sera should be assayed as a negative control. Prepare antibody dilutions in cone- or round-bottom microtiter plates before adding them to antigen-coated plates.

Sources of appropriate antibodies and conjugates can be found in Linscott's Directory of Immunological and Biological Reagents.

Test antigen solution

0.2 to 10 µg/ml antigen, purified or partially purified in PBSN; store at 4°C

Wash buffer

Hanks balanced salt solution (HBSS; APPENDIX 2)

1% fetal calf serum (FCS; heat-inactivated 60 min, 56°C)

0.05% NaN₃

Store at 4°C

COMMENTARY

Background Information

Since their first description in 1971 (Engvall and Perlman), ELISAs have become the system of choice when assaying soluble antigens and antibodies. Factors that have contributed to their success include their sensitivity, the long shelf-life of the reagents (alkaline phosphatase conjugates typically lose only 5% to 10% of their activity per year), the lack of radiation hazards, the ease of preparation of the reagents, the speed and reproducibility of the assays, and the variety of ELISA formats that can be generated with a few well-chosen reagents. Additionally, no sophisticated equipment is necessary for many ELISA applications, including screening hybridoma supernatants for specific antibodies and screening biological fluids for antigen content.

The ELISAs described here combine the special properties of antigen-antibody interactions with simple phase separations to produce powerful assays for detecting biological molecules. The multivalency of antibodies can result in the formation of long-lived antigen-antibody complexes, thus allowing long peri-

ods of time during which such complexes can be measured. By designing an assay so that a capture reagent initiates the binding of antigen-antibody complexes and enzyme conjugates onto a solid phase, the unbound reagents can be easily and rapidly separated from the solid phase. The solid phase is washed and the amount of bound conjugate is visualized by incubating the solid phase with a substrate that forms a detectable product when hydrolyzed by the bound enzyme. ELISAs are similar in principle to radioimmunoassays, except that the radioactive label is replaced by an enzyme conjugate.

A number of different enzymes have been successfully used in ELISAs, including alkaline phosphatase, horseradish peroxidase, β-galactosidase, glucoamylase, and urease. Alkaline phosphatase—perhaps the most widely used conjugated enzyme—is recommended because of its rapid catalytic rate, excellent intrinsic stability, availability, ease of conjugation, and resistance to inactivation by common laboratory reagents. Additionally, the substrates of alkaline phosphatase are nontoxic

Double-Immunodiffusion Assay for Detecting Specific Antibodies

UNIT 2.3

BASIC PROTOCOL

Double immunodiffusion is a simple gel-based assay for detecting antigen-specific antibodies. Analytical agar gels are poured onto microscope slides that have been precoated with agar. Small wells are punched 0.5- to 0.75-cm apart in the analytical gel. Antigen and antibody solutions are placed in adjacent wells and allowed to diffuse into the gel for 6 to 48 hr. As antibody and antigen form diffusion gradients that cross each other, a line of immunoprecipitation may form between the wells, indicating the presence of specific antibodies. The gel is then stained and destained until precipitin lines are maximally visible.

Materials

Noble agar (Difco)
PBS (APPENDIX 2) containing 0.05% NaN_3 (PBSN)
4% PEG 6000 (J.T. Baker) in PBSN, prewarmed to 56°C (store at room temperature)
1 mg/ml antigen
Antisera
Staining solution
Destaining solution
2 × 3-in. microscope slides, precleaned
Boiling and 56°C water baths
50°C oven
Template (see Fig. 2.3.1)
15-G stainless steel needle (blunt-ended and beveled) or immunodiffusion punch set (EC Apparatus)
10- μl Hamilton syringe
Humidified chamber (enclosed plastic container with moistened tissues; Fig. 3.8.1)
Staining rack and dish
Whatmann 3MM filter paper

Precoat microscope slides with agar

1. Prepare a 0.5% noble agar solution in PBSN and place in a boiling water bath until agar dissolves.
2. Place 2 × 3-in. microscope slides on a level surface and pipet 8 ml of 0.5% melted agar evenly over the surface of each slide. Do not disturb gels until the agar has set.
3. Allow gels to dry 4 hr in a 50°C oven or overnight at room temperature.

A dried agar precoat provides an adhesive base that prevents the analytical agar from separating from the slide during staining and destaining treatments.

Prepare analytical gel

4. Dissolve 2% noble agar in PBSN in a boiling water bath.
5. Place coated microscope slides on a level surface. Cool the 2% melted agar to 56°C. Mix 2% melted agar 1:1 with 56°C PEG solution and pipet 10 ml evenly over each slide. Do not disturb gels until the agar has set.

PEG stabilizes immunoprecipitates and increases their visibility.

6. Place the agar gel over a template and, using a blunt-ended and beveled 15-G needle or an immunodiffusion punch set, carefully punch wells to accommodate all antigen and antisera solutions to be tested (see Fig. 2.3.1).

Antibody Detection
and Preparation

2.3.1

7. Remove agar plugs using a Pasteur pipet attached to a vacuum line.

Use a weak vacuum to remove the agar plugs, taking care not to disturb the surrounding agar field.

Load the gel

8. Prepare three antigen samples to be tested against undiluted antisera—one should be ~1 mg/ml, and the other two should be ~500 and 250 µg/ml (prepared by diluting 1 mg/ml antigen 1:1 serially with PBSN). Using a 10-µl Hamilton syringe or pipettor, fill the central wells with an antigen sample and surrounding wells with antisera (the wells hold 5 to 10 µl). Maintain slides on a level surface, and allow samples to diffuse into the gel.

When screening antisera, multiple dilutions of antigen should be tested against all antisera. To increase the amount of reagent loaded, wells can be filled 2 or 3 times. After the liquid is absorbed into the gel (~5 to 10 min), the wells may be refilled with antigen solution.

9. Place loaded gels in a humidified chamber and incubate 48 hr at room temperature. Examine the gels and score for precipitin lines at 6, 24, and 48 hr.

Gels should not be in direct contact with the moistened tissues in the humidity chamber.

Wash and stain the gels

10. Place gels in a staining rack. Place rack in a staining dish filled with PBSN and incubate 24 hr at room temperature with gentle stirring using a magnetic stirbar.

Washing is done in steps 10 to 12 to remove proteins that are not precipitated.

11. Replace PBSN with fresh solution and incubate 24 hr at room temperature.
12. Remove salt by replacing PBSN with water. Incubate 4 hr at room temperature.
13. Remove gels from the staining rack and place face-up on a flat surface. Dry the gels by covering with 3MM filter paper and leaving overnight at room temperature.
14. Place dry gels in a staining rack and immerse 10 min in staining solution at room temperature.

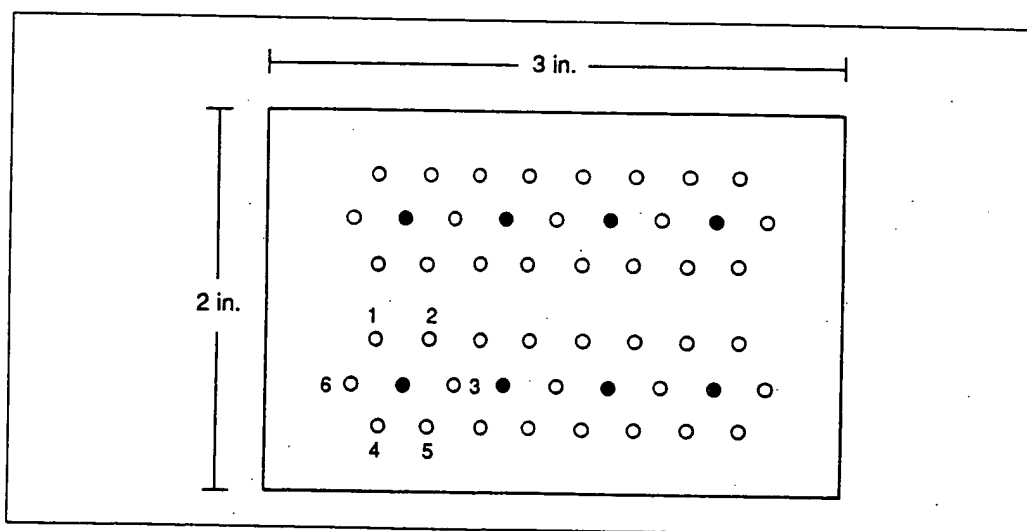


Figure 2.3.1 Double-diffusion template consisting of eight partially overlapping hexagonal arrays distributed around eight central wells. Central wells are represented by dark circles. A numbered set of wells arranged hexagonally around a central well is shown.

15. Destain by immersing gels 4 min in destaining solution. Repeat until precipitin lines are maximally visible and the background staining is negligible.
16. Air dry the gels at room temperature.

REAGENTS AND SOLUTIONS

Destaining solution

- 15% (vol/vol) ethanol
- 5% (vol/vol) glacial acetic acid
- 80% H₂O
- Store at room temperature

Staining solution

- 0.5% (wt/vol) Coomassie Brilliant Blue R-250
- 40% (vol/vol) ethanol
- 10% (vol/vol) glacial acetic acid
- 50% H₂O
- Store at room temperature

COMMENTARY

Background Information

Gel-diffusion techniques, among the earliest methods for detecting specific antibodies and for measuring antigenicity (Ouchterlony and Nilsson, 1986), are still useful methods for detecting specific antibodies. However, they do require high concentrations of both antigen and antibody and are relatively insensitive to antibodies with low affinities. Recently, a silver-staining technique has been described that increases the sensitivity of double-diffusion assays 10 to 100 times (Rochu et al., 1989).

Double immunodiffusion owes its success to the unique nature of antibody-antigen interactions. When polyvalent antibodies with moderate-to-high intrinsic affinities are mixed with antigen at the right ratio—called the zone of equivalence—lattices of antibody-antigen complexes form and precipitate out of solution. When gradients of antigen and antibody are established by diffusion from adjacent wells in a bed of agar, a line of practically insoluble precipitation forms at the equivalence zone (precipitin lines).

Critical Parameters

In this assay the initial antigen and antibody concentrations must be able to support the formation of equivalence zones. For this reason, three different antigen concentrations are recommended. If no lines of precipitation are observed, more sensitive techniques, (e.g., ELISAs) should be considered.

Anticipated Results

Double-diffusion assays in which the immunoprecipitates are stained with Coomassie Brilliant Blue can be sensitive to as little as 25 µg/ml of specific antibody. In the absence of staining, the assay is sensitive to ~100 µg/ml of specific antibody. Details of the precipitation patterns (double precipitin lines, spurs, and lines of identity) can reveal information about the antigenic specificities of various antisera and information about the structure of the antigen (Ouchterlony and Nilsson, 1986). Figure 2.3.2 illustrates typical results.

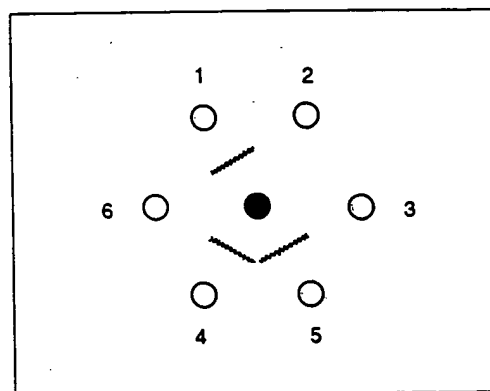


Figure 2.3.2 Typical results of a double-immunodiffusion assay. Wells 1, 4, and 5 are positive for reactive antisera, while wells 2, 3, and 6 are negative.

Time Considerations

The higher the titer of specific antibody, the more rapid the precipitation. Precipitation may begin in some systems within a few hours, while in others it may take 24 to 48 hr to complete.

Literature Cited

Ouchterlony, O. and Nilsson, L.-A. 1986. Immunodiffusion and immunoelectrophoresis. *In* Handbook of Experimental Immunology, Vol. 1: Immunochimistry (D.M. Weir, L.A. Herzenberg, C. Blackwell, and L.A. Herzenberg, eds.) pp. 32.1-32.50. Blackwell, Oxford.

Rochu, D., Crespeau, H., Fine, A., and Fine, J.-M. 1989. A sensitive double-diffusion microassay suitable for the detection of idiotype-anti-idiotypic precipitates. *J. Immunol. Methods* 118:67-71.

Key Reference

Ouchterlony and Nilsson, 1986. See above.

Contains a detailed description of immunodiffusion techniques and provides detailed interpretations of various patterns of immunoprecipitation observed in double-immunodiffusion experiments.

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NCBI Glossary



Other Glossaries

[BLAST Glossary](#)

[Talking Glossary \(NHGRI\)](#)

[NCBI Handbook Glossary](#)

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Abstract Syntax Notation 1 (ASN.1)

ASN.1 is a standard data description language that is used for encoding structured data. ASN.1 allows both the content and the structure of the data to be read by and exchanged between a variety of computer programs and platforms. ASN.1 is the language used to store and manipulate data at the NCBI. All NCBI software reads and writes ASN.1.

Accession Number

The accession number is the most general identifier used in the NCBI sequence databases. This is the identifier that should be used when citing a database record in a publication. The accession number points to a sequence record and does not change when the sequence is modified. In the Entrez system, using the accession number as a query will retrieve the most recent version of the record. The update history of a particular sequence record is tracked by the accession.version number. Changes in version numbers occur only when the actual sequence of a record has been modified and do not reflect any changes in the annotation. The specific version of a record is also tracked by another identifier that is mainly for internal NCBI use called the GI number.

Algorithm

An algorithm is a formal stepwise path to solving a problem, for example the problem of finding high-scoring local alignments between two sequences. Algorithms are the basis of computer programs.

Alignment Score

The alignment score is a number assigned to a pairwise or multiple alignment of sequences that provides a numerical value reflecting the quality of the alignment. Alignment scores are usually calculated by referring to some sort of substitution table or alignment scoring matrix and summing the values for each pair or column in the alignment. (See also raw score and bit score). With certain scoring matrices, high scores of local ungapped alignments between two random sequences have the special property of following the extreme value distribution. This property allows a significance level to be assigned to local alignment scores obtained from database searches using such tools as BLAST and FASTA. (See also Expect value.)

Alignment Scoring Matrix

A scoring matrix is a table of values used to assign a numerical score to a pair or column of aligned residues in a sequence alignment. The simplest kind, an identity matrix, assigns a high value for a match and some low, often negative value, for a mismatch. The identity matrix is used in the NCBI's nucleotide-nucleotide BLAST program. Protein alignment scoring matrices are usually more complicated and take into account the relative abundance of the amino acids in real proteins and the observation that some amino acids substitute for each other more readily in related proteins (e.g., Phe and Tyr) and others do not (e.g., Phe and Asp). One way of generating such a matrix is to examine alignments of real proteins that are known to be homologous (see Homolog) and tabulate the substitution frequencies of the various amino acid pairs at all positions. The resulting frequency table is then converted to a log-odds additive matrix by taking the log of the ratio of the observed substitution frequency for a particular pair and the background substitution frequency. The PAM and the BLOSUM series are examples of widely used protein-scoring matrices that are derived in this way. The matrices described above do not take into account differences in substitution frequencies at different positions in the alignments. More sensitive position-specific scoring matrices can also be generated. Scores of local alignments of random sequences derived from these log-odds matrices are described by the extreme value distribution. Thus, significance levels can be assigned to results of database searches with these matrices using tools such as BLAST and FASTA. (See also Expect value.)

Alu

Alus are the most common class of short, interspersed, repetitive element (SINE) in the human genome. Alus may account for more than 10% of the human genome. They appear to be derived from a signal recognition particle pseudogene. The name Alu derives from the fact that these elements usually contain an *AluI* restriction enzyme recognition site.

Assembly

A sequence assembly is a large sequence or ordered set of sequences that may be derived from overlapping smaller sequences and sometimes anchored to a genome or chromosome scale map using information from STS content and other evidence.

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Bacterial Artificial Chromosome (BAC)

A BAC is a large insert cloning vector capable of handling large segments of cloned DNA, typically around 150 kb. BACs can be propagated in laboratory strains of *Escherichia coli*. These vectors are used in the construction of genomic libraries for genome scale sequencing projects including human, mouse, *Arabidopsis*, and rice.

BankIt

BankIt is a Web form for submitting sequences to GenBank.

Basic Local Alignment Search Tool (BLAST)

BLAST is the NCBI's sequence similarity search tool. It finds high-scoring local alignments between a query sequence and nucleotide and protein database sequences. Although BLAST is less sensitive than the complete Smith-Waterman algorithm, it provides a useful compromise between speed and sensitivity, especially for searching large databases. Because BLAST reports back local alignment scores, it provides statistics that may allow biologically interesting alignments to be distinguished from chance alignments.

Bit Score

The bit score represents the information content in a sequence alignment. It is expressed in base 2 log units. The bit score is in essence a normalized score adjusted by database and matrix scaling parameters. Hence, bit scores for different searches may be compared and only the search space size is needed to calculate the significance (Expect value) of the score. The relationship between Expect value (E) and bit score (S') is shown in equation 3 below.

$$E = mn 2^{-S'} \quad (3)$$

BLOSUM Matrix

The BLock SUBstitution Matrices are a set of protein log-odds alignment scoring matrices calculated from substitution frequencies obtained from ungapped multiple alignments of real proteins. Each BLOSUM matrix is identified with a number that indicates the percent identity cut-off for inclusion in that matrix. For example BLOSUM62, includes substitution information for proteins up to 62% identical in the alignment, BLOSUM90 up to 90% identical. Each BLOSUM matrix works best at finding proteins at a particular level of similarity. Hence, BLOSUM90 is better at finding more closely related proteins whereas BLOSUM62 is best at finding more distantly related ones. Experiments have shown that BLOSUM62 also works well at finding similar proteins. For this reason, BLOSUM62 is the default protein scoring matrix for NCBI BLAST.

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Clone

In the molecular sense, a clone is a physical copy of a piece of DNA. The term is most often used to refer to the recombinant cloning vector DNA containing this copy such as a plasmid, BAC, or bacteriophage

DNA that can be propagated in a bacterial or other microbial host.

Cluster

A cluster is a group of sequences associated with each other, usually by some procedure that relies on sequence similarity. Such clusters of sequences are used to produce the UniGene datasets and the clusters of orthologous groups (COGS) dataset.

Clusters of Orthologous Groups (COGs)

A COG is a group of related proteins or groups of proteins (paralogs) from different genomes that are thought to derive from a common ancestral gene. COGs are formed based on sequence similarity using a BLAST-based approach. COGs originally were made for the complete microbial genomes, but the dataset is expanding to include more complex organisms. The COGs data are very useful for annotating genes on microbial genomes and can be used to provide potential functional classification for uncharacterized proteins. (See also paralog and ortholog.)

Cn3D

Cn3D (pronounced "see in three dee") is NCBI's structure viewer. It reads Entrez structure data and renders either single structures or structural alignments from the NCBI's molecular modeling database (MMDB). Cn3D functions as a helper application to the Web browser and will launch automatically when the browser downloads NCBI structure data. Cn3D can also function as a stand-alone viewer and can act as a network client to download structures from NCBI. It also has a built-in BLAST and threading capability and can create sequence alignments to fit similar sequences to known structures.

Conserved Domain Architecture Retrieval Tool (CDART)

CDART provides a graphical browser that allows one to find proteins with a similar domain architecture (content and arrangement) beginning with the results of a CDD search.

Conserved Domain Database (CDD) Search

CDD Search uses reverse position-specific BLAST (RPS-BLAST) to identify conserved domains contained in a protein query. CDD databases are position-specific scoring matrices (PSSMs) created from multiple sequence alignments from three domain databases: SMART, PFAM, and LOAD.

Contig

Contig is short for contiguous sequence. Contigs are assembled overlapping primary sequences. The term contig arises in two different contexts in the NCBI databases. Draft sequences (HTG division) will contain two or more contigs assembled from sequencing reads made from plasmid libraries for that clone. The NCBI also produces contigs made by assembling overlapping GenBank records from large-scale genome projects, such as the human genome project. These contigs are

included in the NCBI RefSeq databases and are assigned accession numbers beginning with the prefix NT_.

Curated Database

A curated database is a derivative database containing molecular records that are compiled and edited from primary molecular data by experts who maintain and are responsible for the content of the records. The Swiss-Prot database is an important example of curated protein sequence database. The NCBI produces a curated non-redundant RefSeq dataset of transcripts and proteins for important organisms.

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Derivative Database

In molecular biology, a derivative database contains information derived and compiled from primary molecular data but includes some type of additional information provided by expert curators or automated computational procedures.

DNA Databank of Japan (DDBJ)

A primary nucleotide sequence database that is maintained as part of the Center for Information Biology and DNA Data Bank of Japan (CIB/DDBJ) under the National Institute of Genetics (NIG) in Japan. DDBJ began accepting DNA sequence submissions in 1986 and is a part of the International Nucleotide Sequence Database Collaboration that also includes GenBank and the EMBL nucleotide sequence database.

Domain

A domain is a discrete structural unit of a protein. In principle, protein domains are capable of folding independently from the rest of the protein. Domains can often be identified by non-structural approaches based on conserved amino acid sequences. The NCBI's CDD-search uses information from curated multiple sequence alignments to identify domains in protein sequences.

Draft Sequence

Draft sequence is unfinished genomic or cDNA sequence. See HTG and HTC.

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Electronic PCR (e-PCR)

e-PCR is an analysis tool that tests a DNA sequence for the presence of sequence tagged sites (STSs). e-PCR looks for STSs in DNA sequences

sequences by searching for subsequences that closely match the PCR primers and have the correct order, orientation, and spacing that they could plausibly prime the amplification of a PCR product of the correct length.

European Molecular Biology Laboratory (EMBL) Database

A nucleotide sequence database produced and maintained at the European Bioinformatics Institute (EBI) in Hinxton, UK, that collaborates with GenBank and the DNA Database of Japan (DDBJ) to form the International Nucleotide Sequence Database Collaboration.

Ensembl

Ensembl is a joint project between EBI-EMBL and the Sanger Institute to provide automatic annotation of eukaryotic genomes.

Entrez

Entrez is an integrated search and retrieval system that integrates information from various databases at NCBI, including nucleotide and protein sequences, 3D structures and structural domains, genomes, variation data (SNPs), gene expression data, genetic mapping data, population studies, OMIM, taxonomy, books online, and the biomedical literature.

European Bioinformatics Institute (EBI)

A non-profit academic organization that performs research in bioinformatics and maintains the EMBL nucleotide sequence database.

Evidence Viewer

A feature within the human genome Map Viewer that provides a graphical display of the molecular evidence supporting the existence of a gene model. ev displays reference sequences, GenBank mRNAs, annotated known or potential transcripts, and ESTs that align to the genomic area of interest.

Expect Value (E-value)

In BLAST statistics, the Expect value is the number of alignments with a particular score, or a better score, that are expected to occur by chance when comparing two random sequences. The relationship between expect value and alignment score is given by equation 1

$$E = Kmn e^{-\lambda S} \quad (1)$$

In Equation 1, e is the base of the natural logarithm scale, n and m are the lengths of the two sequences, essentially the search space size for database searching, and K and λ are scaling factors for the search space and the scoring system, respectively. The bit score incorporates λ and K so that scores can be meaningfully compared when different databases and scoring systems are used.

Expressed Sequence Tag (EST)

A short (300-1000 nucleotide), single-pass, single-read DNA sequence derived from a randomly picked cDNA clone. EST sequences comprise the largest GenBank division. There are numerous high-throughput sequencing projects that continue to produce large numbers of EST sequences for important organisms. Many ESTs are classified into gene-specific clusters in the UniGene data set.

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FASTA

A sequence similarity search tool developed by William Pearson and David Lipman. The term FASTA is also used to identify a text format for sequences that is widely used. A FASTA-formatted sequence file may contain multiple sequences. Each sequence in the file is identified by a single line title preceded by the greater than sign (">"). Example.

Feature Table

The feature table is the portion of the GenBank record that provides information about the biological features that have been annotated on the nucleotide sequence, including coding and non-coding regions, genes, variations, and sequence tagged sites. The International Sequence Database Collaboration produces a document describing and identifying allowed features on GenBank, DDBJ, and EMBL records.

File Transfer Protocol (FTP)

FTP is a standard Internet protocol used to transfer files to and from a remote network site.

Fluorescence in Situ Hybridization (FISH) map

A FISH map is a cytogenetic map derived from the localization of fluorescently-labeled probes to chromosomes. Genes are mapped according to their cytogenetic (band position) location on the chromosome.

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GenBank

GenBank is a primary nucleotide sequence database produced and maintained at the National Center for Biotechnology Information (NCBI) at the National Institutes of Health (NIH) in Bethesda, MD, USA. GenBank collaborates with EMBL and DDBJ to form the International Nucleotide Sequence Database Collaboration.

GenBank Division

GenBank divisions are partitions of the GenBank data into categories based on the origin of the sequence. At first the GenBank divisions were established so that one division would be one file in the GenBank distribution. However, the number of GenBank divisions has not kept pace with the growth of the sequence data; the EST division now has over 150 files. There are currently 17 GenBank divisions.

GenBank Flatfile Format

This is the format of the sequence records in the GenBank flatfile release. This is a text-only format containing multiple entries or records. Each record in the large text file, also called a flatfile, begins with a LOCUS line and ends with a single line consisting of a pair of forward slashes ("//"). The term "GenBank format" is often used to refer to the format of individual records within the flatfile. Each record contains a header containing the database identifiers, the title of the record, references, and submitter information. The header is followed by the feature table and then the sequence itself. The GenBank flatfile is described in detail in the GenBank release notes. In the Entrez system, the GenBank format is the default display format for non-bulk sequence entries.

Gene Expression Omnibus (GEO)

GEO is a primary database at the NCBI that is an archived repository for gene expression data derived from different experimental platforms.

Gene Model

A gene model is a mapping of gene features such as coding regions and exon intron boundaries onto the the genomic DNA of an organism. Gene models typically provide a predicted transcript and protein sequence. A simple kind of gene model can be made by aligning an expressed sequence (cDNA) to the genomic DNA sequence. More precise exon intron boundaries can be identified by constraining the aligned segments using consensus splicing signals. This type of alignment-based gene model is used to generate many of the NCBI RefSeq model transcripts for higher genomes. Gene features can also be predicted computationally in the absence of aligned expressed sequences. The simplest candidate gene predictions can be made on microbial genomic DNA by searching for long open reading frames. Database sequence similarity searches with the predicted translations of these ORFs are used to support these gene predictions. Computational gene prediction in higher eukaryotic genomes is complicated by the interruption of gene coding regions by intronic sequences. There are a number of methods that are used in eukaryotic gene prediction. The NCBI uses the program GenomeScan to annotate putative genes on the human, mouse and rat genomes.

Genetic Linkage Map

A linkage map is an ordered display of genetic information referenced to linkage groups (ultimately chromosomes) in a genome. The mapping units (centiMorgans) are based on recombination frequency between various polymorphic markers traced through a pedigree. One centiMorgan equals one recombination event in 100 meioses.

Genetics Computer Group (GCG)

The GCG is a bioinformatics software development group, originally at the Department of Genetics at the University of Wisconsin, then later existing as a private company, and merging with Oxford Molecular, MSI and Synopsis to collectively form Accelrys. GCG is widely known for its sequence analysis software package properly known as the Wisconsin Package. The initials GCG have been widely used as a synonym for that package.

Genome Survey Sequence (GSS)

GSS sequences comprise a bulk sequence division of GenBank. GSS sequences are in essence the genomic equivalent of the ESTs. The GSS division contains first pass, single reads of genomic DNA. Typical GSS records are initial sequencing surveys and end reads of large insert clones from genomic libraries, exon-trapped genomic sequences and Alu PCR sequences.

GenomeScan

GenomeScan is gene prediction program (algorithm) developed by Christopher Burge at the Massachusetts Institute of Technology. This is the algorithm used at the NCBI to produce gene models for higher genomes.

GI Number

The GI number is an identifier assigned to all sequences at the NCBI. The GI number points to a specific version of a sequence record. This identifier is largely superceded by the accession.version number for outside users. GI stands for GenInfo, a database system at NCBI that preceded the Entrez system.

Global Alignment

A global alignment is a sequence alignment that extends the full-length of the sequences that are being compared. Global alignment procedures usually will produce an alignment that includes the entire length of all sequences including regions that are not similar, and can be made to produce meaningless alignments between unrelated sequences. Compare with local alignment.

Golden Path

The Golden Path refers to the human and mouse genome annotation and assembly projects at the University of California Santa Cruz (UCSC).

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High Throughput Genomic Sequence (HTG)

HTG sequences comprise a Genbank division containing unfinished

genomic sequence. HTG records typically are incomplete assemblies sequences of BAC or other large insert clones. GenBank recognizes four stages of completion (phases) for these sequences. Phase 0 records contain one or a few single pass reads of a given genomic clone. Phase 1 records contain two or more assembled contigs of the sequence data; however the contigs are unordered and unoriented and there are still gaps in the sequence. Phase 2 records also contain two or more contigs with gaps, but the order and orientation are known. Once the sequence gaps are resolved, and there is enough sequence coverage to give an accuracy of 99.99%, the record moves to phase 3 and leaves the HTG division for the appropriate taxonomic GenBank division; a human sequence would move to the pirmate division (PRI), a mouse sequence to the rodent division (ROD).

High Throughput cDNA (HTC)

HTC is a GenBank division containing draft cDNA sequences. HTC records are similar to ESTs, but often contain more information. Unlike ESTs but like the genomic draft (HTG) records, HTC sequences may be updated with additional sequence data and move to the appropriate traditional division of GenBank.

Homologue

Two biological entities (structures or molecule) are said to be homologues (or are homologous) if it is thought that they descend from a common ancestral structure or molecule. Correspondong body parts and genes in different or the same species can be homologous. The term has often been extended to include sequences as well. However it is incorrect to report a relative homology or percent homology as is sometimes said of sequences; genes or sequences are either homologous or they are not. See also orthologue and paralogue

Human Genome Nomenclature Committee

The HGNC is a non-profit organization located at the University College London that assigns authoritative and unique gene names and symbols for all known human genes.

Human Mouse Homology Maps

The human mouse homology maps show the syntenic chromosome regions between the two organisms and allow the corresponding sequences and other related information to be retrieved from one organism given a gene or map location in the other. The data used to construct these homology maps are derived from UCSC and NCBI human genome assemblies and the mouse MGD genome map and Whitehead/MRC radiation hybrid maps.

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International Sequence Database Collaboration (ISDC)

The ISDC involves the three major primary nucleotide sequence repositories GenBank, the DDBJ (DNA Data Bank of Japan), and the EMBL (European Molecular Biology Laboratory) databases. Each database has its own set of submission and retrieval tools, but the three exchange data daily and have shared standards for sequence submission and annotation. All three share data so that all contain the same set of sequence data.

Interspersed Repeats

Interspersed repetitive sequences are primarily degenerate copies of transposable elements - also called mobile elements - that, in humans, comprise over a third of the genome. The most common mobile elements are LINEs and SINEs (long and short interspersed nuclear elements, respectively). The Alu families of repeats are the primary SINEs in primates.

LINE

Long interspersed nuclear elements are a class of transposable element, also called an interspersed repeat. These constitute about 20% of the human genome. A typical LINE is 6KB long and encodes a reverse transcriptase and a DNA-nick-looping enzyme, allowing it to move about the genome autonomously. LINEs are also called non-LTR retrotransposons.

LinkOut

LinkOut is registry service to create links from specific articles, journals, or biological data in Entrez to resources on external web sites. Third parties can provide a URL, resource name, brief description of their web site, and specification of the NCBI data from which they would like to establish links.

LOAD

LOAD is the library of ancient domains, a small number of conserved domain alignments that add to the position specific scoring matrices (PSSMs or profiles) in the Conserved Domain Database (CDD) at NCBI. The majority of domains in CDD come from the databases SMART, Simple Modular Architecture Research Tool, and Pfam.

Local Alignment

A local alignment is a high scoring alignment between sub-sequences of two or more longer sequences. Unlike a global alignment, there may be multiple high scoring local alignments between sequences. Local alignments are useful for database searches because their scores can be used to assess the biological significance of the alignments found. (See also Alignment Score and Expect Value.) Local alignments are produced by the popular sequence similarity search tools BLAST and FASTA.

LocusLink

LocusLink is an NCBI resource that provides a single query interface to curated sequence and descriptive information about genetic loci. It is a good place to begin a search for information about a particular gene. LocusLink currently contains human, mouse, rat, zebrafish, fruit fly and HIV-1 loci..

Low Complexity Sequence

Low complexity sequence is a region of amino acid or nucleotide sequence with a biased residue composition. Low complexity sequence includes homopolymeric runs, short-period repeats, and some subtler over-representation of one or a few residues. Such sequences often look very redundant, for example the protein sequence PADPPPDPPPP or the nucleotide sequence AAATTTAAAAAT. Low-complexity regions can result in misleading high scores in sequence similarity searches. These scores reflect compositional bias rather than significant position-by-position alignment. Filter programs are usually used to eliminate these potentially confusing matches from sequence similarity search results. The NCBI BLAST programs used filters that replace low complexity regions in the query sequence with an anonymous residue (n for nucleic acid, X for amino acid) These regions are thus effectively removed from the search because these anonymous residue are treated as mismatches by the BLAST programs.

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Map Viewer

The Map Viewer is a software component of the NCBI Entrez Genomes that provides special browsing capabilities for genomes of higher organisms. It allows one to view and search an organism's complete genome, display chromosome maps, and zoom into progressively greater levels of detail, down to the sequence data. If multiple maps are available for a chromosome, it displays them aligned to each other based on shared marker and gene names, and, for the sequence maps, based on a common sequence coordinate system. The number and types of available maps vary by organism, but include maps for: genes, contigs, BAC tiling path, STSs, FISH mapped clones, ESTs, GenomeScan models, and SNPs.

MEDLINE

MEDLINE is the NLM's premier bibliographic database covering the fields of medicine, nursing, dentistry, veterinary medicine, the health care system, and the preclinical sciences. MEDLINE contains bibliographic citations and author abstracts from more than 4,600 biomedical journals published in the United States and 70 other countries. The file contains over 11 million citations dating back to mid-1960. Coverage is worldwide, but most records are from English-language sources or have English abstracts. MEDLINE is included in PubMed, which contains additional citations.

MegaBLAST

MegaBLAST is a local pairwise nucleotide alignment tool that is optimized for finding long alignments between nearly identical sequences. MegaBLAST is most useful for comparing sequences from the same species, and is particularly suited to such tasks as clustering ESTs, aligning genomic clones or aligning cDNA sequences and genomic DNA. MegaBLAST can be up to 10 times faster than many standard sequence similarity programs, including standard nucleotide-nucleotide BLAST. It also efficiently handles much longer DNA sequences. MegaBLAST is the only BLAST program on the NCBI's web site that can perform batch searches.

Model Maker

Model Maker is a tool associated with the Map Viewer that allows one to view the evidence (mRNAs, ESTs, and gene predictions) that was aligned to assembled genomic sequence in order to build a gene model. Model Maker also allows editing the model by selecting or removing putative exons. Model Maker can then display the mRNA sequence and potential ORFs for the edited model, and save the mRNA sequence data for use in other programs. Model Maker is accessible from sequence maps displayed in the Map Viewer. To see an example, follow the "mm" link beside any gene annotated on the human "Gene_Sequence" map in the Map Viewer.

Molecular Modeling Database (MMDB)

NCBI's structure database, MMDB, contains experimentally determined, three-dimensional, biomolecular structures obtained from the Protein DataBank (PDB); the PDB's theoretical models are not imported. MMDB was designed for flexibility, and as such, is capable of archiving conventional structural data as well as future descriptions of biomolecules, such as those generated by electron microscopy (surface models). Most 3D-structure data are obtained from X-ray crystallography and NMR-spectroscopy.

Motif

A motif is a short, well-conserved nucleotide or amino acid sequence that represents a minimal functional domain. It is often a consensus for several aligned sequences. The PROSITE database is a popular collection of protein motifs, including motifs for enzyme catalytic sites, prosthetic group attachment sites (heme, biotin, etc), and regions involved in binding another protein. Examples of DNA motifs are transcription factor binding sites.

The National Center for Biotechnology Information (NCBI)

The NCBI is a division of National Library of Medicine at the National Institutes of Health in Bethesda, MD. The NCBI was established in 1988 to create automated systems for storing and analyzing knowledge about molecular biology, biochemistry, and genetics; to support the use of such databases and software by the scientific community; to coordinate efforts to gather biotechnology information both nationally and internationally; and to perform research in computational biology. Currently the NCBI maintains the GenBank database along with several related databases.

The National Institute of Genetics (NIG)

The National Institute of Genetics (NIG) was established in 1949 in Mishima, Japan and reorganized in 1988 as an inter-university research institute in genetics. The Institute currently provides graduate education in genetics and also maintains the DNA Data Bank of Japan.

Nonredundant (nr)

Nonredundant is a term used to describe nucleotide or amino acid sequence databases that contain only one copy of each unique sequence. Non-redundant databases have the advantage of smaller size and, therefore, shorter search times and more meaningful statistics. The default database on most protein BLAST web pages is labeled "nr". This is a nonredundant database where multiple copies of the same sequence such as the corresponding sequences of the same protein from SWISS-PROT, PIR, and GenPept, are combined to make one sequence entry. The default nucleotide database on the standard nucleotide-nucleotide BLAST web page is also labeled "nr", but is no longer a nonredundant database.

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Online Mendelian Inheritance in Man (OMIM)

OMIM is a catalog of human genes and genetic disorders authored and edited by Dr. Victor A. McKusick and his colleagues at Johns Hopkins and elsewhere, and developed for the World Wide Web by NCBI. The database contains textual information, references, and copious links to MEDLINE and sequence records in the NCBI's Entrez system, plus links to additional related resources at NCBI and elsewhere.

Open Reading Frame (ORF)

An ORF is a DNA (or mRNA) sequence that is potentially able to encode a polypeptide. ORFs begin with a start codon (ATG) and are read in triplets until they end with a STOP codon (TAA, TGA, or TAG in the standard code). The NCBI ORF finder is useful for identifying ORFs in cDNA or in intron-less genomic DNA.

Orthologue

Orthologues are genes derived from a common ancestor through vertical descent. This is often stated as the same gene in different species. In contrast, paralogs are genes within the same genome that have evolved by duplication.

The hemoglobin genes are a good example. Two separate genes (proteins) make up the molecule hemoglobin (alpha and beta). The alpha and beta DNA sequences are very similar and it is believed that they arose from duplication of a single gene, followed by separate evolution in each of the sequences. Alpha and beta are considered paralogs. Alpha hemoglobins in different species are considered orthologs.

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PAM Matrix

The original Percent Accepted Mutation scoring matrix (see M.O. Dayhoff, ed., 1978, Atlas of Protein Sequence and Structure, Vol 15) was derived from observing how often different amino acids replace other amino acids in evolution, and was based on a relatively small dataset of 1,572 changes in 71 groups of closely related proteins. Further, matrix values are based on the model that one sequence is derived from the other by a series of independent mutations, each changing one amino acid in the first sequence to another amino acid in the second. PAM250 was a very popular matrix, but is often now replaced by the BLOSUM series of matrices, particularly when looking for more distantly related proteins. Lower number PAM matrices correspond roughly to higher numbered BLOSUM matrices.

Paralog

Paralogs are usually described as genes within the same genome that have evolved by duplication. See Ortholog.

PFAM database

Pfam is a database of conserved protein regions or domains. It is one of three databases that make up the NCBI's Conserved Domain Database (CDD). The other two are SMART and LOAD.

PopSet

A PopSet is a set of DNA sequences that have been collected to analyze the evolutionary relatedness of a population. The population could originate from different members of the same species, or from organisms from different species. They are submitted to GenBank via the program Sequin, often as a sequence alignment.

Position Hit Initiated BLAST (PHI-BLAST)

PHI-BLAST is a variation of BLAST that is designed to search for proteins that both contain a pattern specified by the user, and are similar to the query sequence in the vicinity of the pattern. This dual requirement is intended to reduce the number of database hits that contain the pattern and are likely to have no true homology to the query.

Position Specific Iterated BLAST (PSI-BLAST)

PSI-BLAST is a derivative of protein-protein BLAST that is more sensitive because it incorporates position specific substitution rates in the scoring system. This makes PSI-BLAST useful for finding very distantly related proteins. PSI-BLAST works by first generating a position specific score matrix (PSSM) from the sequences found from a standard BLAST search. The database is then searched with the PSSM. PSI-BLAST can be run in multiple iterations with a new PSSM being made from the the new information collected from the previous search.

Position Specific Scoring Matrix (PSSM)

A PSSM is an alignment scoring matrix that provides substitution scores for each position in a protein sequence. PSSMs are often based upon the frequencies of each amino acid substitution at each position of protein sequence alignment. This gives rise to scoring matrix that has the length of the alignment as one dimension and the possible substitutions in the other. In a PSSM a particular substitution, for example Ser substituting for Thr, can have a different score at different positions in the alignment. This is in contrast to a position independent matrix like BLOSUM62, where the Ser Thr substitution gets the same score no matter where it occurs in the protein. PSSMs are more realistic models for related protein sequences since substitution rates are expected to vary across the length of a protein; some aligned positions, such as the active site residues, are more important than others.

Positives

In the context of alignments displayed in BLAST output, positives are those non-identical substitutions that receive a positive score in the underlying scoring matrix, BLOSUM62 by default. Most often, positives indicate a conservative substitution or substitutions that are often observed in related proteins.

Primary Database

A primary sequence database contains sequences submitted by the researchers who originally produced the data. In primary sequence databases the submitters of the sequence control the contents and disposition of the data. GenBank is an example of a primary database. The content, accuracy and updating of GenBank sequences is largely the responsibility of the submitter. This is in contrast to a curated database, such as RefSeq or SWISS-PROT, where additional information is added to each record by the staff maintaining the

database.

ProbeSet

ProbeSet is a by experiment view of NCBI's Gene Expression Omnibus (GEO), which is a gene expression and hybridization array repository. ProbeSet is intended to facilitate searches of the GEO database, and link the search results to internal and external resources where possible.

ProtEST

Protein matches for ESTs (ProtEST) are the best protein matches to translations of EST sequences in UniGene. The nucleotide sequences (mRNAs as well as ESTs) are matched with possible translational products through sequence comparison using BLASTX with an expect value of 1×10^{-6} . The sequences are compared with proteins from eight organisms and the best match in each organism is recorded. UniGene nucleotide sequences can thus have up to eight matches in ProtEST. In order to exclude proteins sequences that are strictly conceptual translations or models, the proteins used in ProtEST are those originating from the structural databases SwissProt, PIR, PDB or PRF.

Protein Data Bank (PDB)

PDB is the repository for the processing and distribution of 3-D biological macromolecular structure data. As of April, 2002, PDB contained almost 18,000 structures, including more than 1,000 nucleic acids and 400 theoretical models. Except for theoretical models, the PDB data are used to produce the NCBI's structure database, MMDB and are included in the default BLAST databases("nr").

Protein Information Resource (PIR)

PIR is a curated protein sequence database produced and maintained by the National Biomedical Research Foundation at Georgetown University in Washington, D.C. PIR protein sequences are included in BLAST "nr" database and in the Entrez protein system. PIR contains more than 200,000 entries.

Protein Resource Foundation (PRF)

PRF is a protein sequence database maintained in Osaka, Japan, and is one of the protein databases included in BLAST "nr" database searches and in the Entrez protein system. Release 84, March 2002, included 195,660 entries.

PubMed

PubMed, a service of the National Library of Medicine, provides access to over 11 million MEDLINE citations, from more than 4,300 biomedical journals published in the United States and 70 other countries. Citations cover the fields of medicine, nursing, dentistry, veterinary medicine, the health care system, and the preclinical

sciences; and date back to mid-1960. PubMed includes additional life science journals not found in MEDLINE, as well as links to many sites providing full text articles and other related resources.

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Radiation Hybrid (RH) map

A radiation hybrid map is a STS-based physical genome map produced by first breaking chromosomes of a donor cell line with a lethal dose of radiation, and then rescuing the cells by fusion with a recipient cell line. Distances between markers are measured in centirays (cR), with 1 cR representing a 1% probability that a break occurred between two markers.

RasMol

RasMol is a structure rendering software package produced at the University of Massachusetts. RasMol interprets the native format of structure files from PDB.

Raw Score

A raw score in BLAST output is the non-normalized score of an alignment of a query and target sequence. The raw score is derived directly from the scoring matrix by summing the individual substitution scores of the aligned residues in the alignment. For gapped BLAST the raw score also includes gap penalties.

Reference SNP

Reference single nucleotide polymorphisms (refSNP) are curated dbSNP records that define a non-redundant set of markers used for annotation of reference genome sequence and integration with other NCBI resources. Each refSNP record provides a summary list of submitter records in dbSNP and a list of external resource and database links.

RefSeq

Reference Sequences are curated nucleotide or protein records developed by NCBI staff. They attempt to summarize the available information about a given sequence and to provide the most reliable sequence information to date. RefSeqs include curated transcripts and proteins, noncoding transcribe RNAs, contig and supercontig assemblies, gene models and chromosome records.

Reverse Position Specific BLAST (RPS-BLAST)

RPS-BLAST is a variation of BLAST in which a protein query sequence is searched against a database of pre-computed Position-Specific Score Matrices as used in PSI-BLAST. This kind of search forms the basis of the CD-Search.

Sequence Alignment

A sequence alignment is a residue by residue comparison of two or more sequences. In a sequence alignment the relative positions of the sequences are adjusted to optimize (usually maximize) the alignment score derived by reference to some scoring matrix. In some cases gaps with associated penalties may be inserted into one or more sequences to optimize the alignment score.

Sequence Tagged Site STS

STS's are sequence records that contain a short sequence of genomic DNA that can be uniquely amplified by the polymerase chain reaction (PCR) using a pair of primers. The primer sequences and PCR conditions are usually included in the record. Sequence tagged sites comprise the STS GenBank division. These markers are used in linkage linkage and radiation hybrid mapping techniques. They are useful for integrating these kinds of mapping data with each other and also with the assembled genomic sequence. The ePCR tool is useful for indentifying known STS markers in a DNA sequence.

Sequin

Sequin is a stand alone application package produced by NCBI that is platform for preparing and annotating sequences for submission to GenBank.

Serial Analysis of Gene Expression(SAGE)

SAGE is an experimental method of generating a cDNA library that contains concatenated short (usually ten base) fragments called tags of all cDNA species present in library. These tags may be counted to give a quantitative measure of gene expression in the library. The NCBI SAGE Map resources match SAGE tag sequences to UniGene cluster to identify genes expressed in SAGE libraries and provide several mechanisms for exploring relative expression patterns in SAGE libraries..

Shotgun Sequencing

Shotgun sequencing is a sequencing method in which a large genomic clone is broken into small segments that are then subcloned and randomly sequenced. Once enough random clones have been sequenced, these random sub-sequences are then assembled to establish the large insert sequence. In some cases an entire genome may be fragmented and cloned into small insert vectors without first being cloned and arrayed in large insert vectors. This latter technique is called whole genome shotgun sequencing and has been used successfully with many smaller genomes and has provided important preliminary assemblies for the human, mouse and rice genomes.

SINE

SINEs (Short Interspersed Repeats) are transposable repeat elements in

the human genome that are typically 100-400 bp, harbor an internal polymerase III promoter, and encode no proteins.

Single Nucleotide Polymorphism (SNP)

Strictly speaking a SNP is a variation or polymorphism in the genome sequence involving a single nucleotide position. The NCBI maintains dbSNP as a primary repository of SNP data. The SNP data at the NCBI also includes some variations involving multiple positions such as repeat polymorphisms.

Spectral Karyotyping and Comparative Genomic Hybridization Database (SKY/CHG database)

SKY/CHG is a repository of publicly submitted data from SKY and CGH, which are complementary fluorescent molecular cytogenetic techniques. SKY facilitates identification of chromosomal aberrations; CGH can be used to generate a map of DNA copy number changes in tumor genomes.

SMART

SMART (Simple Modular Architecture Retrieval Tool) is a database of conserved domains that allows automatic identification and annotation of domains in user-supplied protein sequences. The SMART data are used to create one of the sets of PSSMs used in the CD-Search.

Smith Waterman algorithm

The Smith-Waterman algorithm is a local alignment computational protocol that uses dynamic programming to find all possible high-scoring local alignments between a pair of sequences. This is the most sensitive local alignment algorithm but is computationally too expensive to be generally useful for high throughput searches of large sequence databases. The BLAST and FASTA programs are generally used in these kinds of applications.

SWISS-PROT

SWISS-PROT is a highly curated database of protein sequences established in 1986 and currently maintained by the Swiss Institute of Bioinformatics and the European Bioinformatics Institute (EBI).

TaxBrowser

The TaxBrowser is an aspect of the Entrez system that allows one to browse sequence, genome and structure records based on the taxonomic classification of the source organism. The tax browser allows access at all levels of the taxonomic hierarchy and can be used to acquire records at any taxonomic node.

TrEMBL

TrEMBL (Translated EMBL) is a derivative protein dataset that is an automatically-annotated supplement to the SWISS-PROT. TrEMBL contains all the translations of coding regions of EMBL nucleotide

sequence entries. The trEMBL data set serves as a source of proteins that may eventually be incorporated into SWISS-PROT.

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UniGene

A database created and maintained at NCBI as an experimental system for automatically partitioning expressed nucleotide sequences into a non-redundant set of gene-oriented clusters. Each UniGene cluster contains sequences that represent a unique gene, as well as related information such as the map location and tissue types in which the gene has been expressed. UniGene is particularly important for reducing the redundancy and complexity of EST data and is an important resource for gene discovery.

UniSTS

A resource created and maintained at NCBI that reports information about Sequence Tagged Sites (STS). For each STS, UniSTS displays the primer sequences, product size, and mapping information, as well as cross references to other NCBI databases.

Vector Alignment Search Tool (VAST)

An algorithm created at NCBI that searches for three-dimensional structures that are geometrically similar to a query structure by first representing the secondary structure elements of each structure as vectors, and then attempting to align these sets of vectors. VAST is used at the NCBI to establish relationships between structures and create structural alignments in the Entrez system.

Word Size

A parameter of the BLAST algorithm that determines the length of the residue segments (either nucleotides or amino acids) into which BLAST partitions the query sequence. The resulting dictionary of "words" is then used to search the selected sequence database.

Yeast Artificial Chromosome (YAC)

A YAC is a functional (self-replicating) artificial chromosome widely used as a vector for genomic clones in sequencing projects involving large genomes. As the name implies, YACs are propagated in yeast (*Saccharomyces*). A typical YAC clone can contain fragments up to ~2 Mb. A major problem with YAC clones is their tendency to rearrange in the host. YAC technology has largely been replaced by BAC cloning vectors.

Questions or Comments?
Write to Peter Cooper